[CONTRIBUTION FROM THE INSTITUTE FOR ENZYME RESEARCH, THE UNIVERSITY OF WISCONSIN, MADISON, WIS.]

Studies on Polynucleotides. XIX.[1] The Specifiic Synthesis of $C_{3'}$–$C_{5'}$ Inter-ribonucleotidic Linkage.[2] A New Approach and its Use in the Synthesis of $C_{3'}$–$C_{5'}$-Linked Uridine Oligonucleotides[3]

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In seeking methods for the protection of 2′-hydroxyl group in ribonucleoside-3′ phosphates by alkali-labile groups, acetylation of uridine-3′ phosphate was studied under a variety of conditions. Formation of 5′-*O*-acetyluridine-2′,3′-cyclic phosphate competed with the formation of the desired 2′,5′-di-*O*-acetyluridine-3′ phosphate. Conditions were found for the exclusive formation of the latter. 5′-*O*-Tetrahydropyranyluridine-3′ phosphate was prepared by partial hydrolysis of 2′,5′-di-*O*-tetrahydropyranyluridine-3′ phosphate. Acetylation of 5′-*O*-tetrahydropyranyluridine-3′ phosphate followed by an acidic treatment and partition chromatography gave 2′-*O*-acetyluridine-3′ phosphate. The latter compound was also prepared *via* 5′-*O*-di-*p*-methoxytrityluridine-3′ phosphate which was obtained by direct reaction of uridine-3′ phosphate with di-*p*-methoxytrityl chloride. In model experiments on the $C_{3'}$–$C_{5'}$ inter-ribonucleotidic linkage, the reaction of 2′,5′-di-*O*-acetyluridine-3′ phosphate and of 2′-*O*-acetyluridine-3′ phosphate with dicyclohexylcarbodiimide in the presence of a large excess of methyl alcohol, followed by ammoniacal treatment, gave, in each case, pure methyl uridine-3′ phosphate in quantitative yield. The condensation of 2′,5′-di-*O*-acetyluridine-3′ phosphate with N,N,2′-*O*,3′-*O*-tetrabenzoyladenosine followed by an alkaline treatment gave uridylyl-(3′ → 5′)-adenosine in 84% yield. The reaction of 2′-*O*-acetyluridine-3′ phosphate with dicyclohexylcarbodiimide in dry pyridine at room temperature followed by an ammoniacal treatment afforded a number of polymeric products, from which uridylyl-(3′ → 5′)-uridine-3′ phosphate (UpUp), uridylyl-(3′ → 5′)-uridylyl-(3′ → 5′)-uridine-3′ phosphate (UpUpUp), cyclo-[uridylyl-(3′ → 5′)-uridylyl-(3′ → 5′)] and the homologous cyclic trinucleotide were isolated pure and characterized. The synthetic oligonucleotides were completely degraded by pancreatic ribonuclease to uridine-3′ phosphate. The present work constitutes the first successful polymerization of a suitably protected ribomononucleotide to form oligonucleotides containing exclusively the $C_{3'}$–$C_{5'}$ inter-ribonucleotidic linkages.

Introduction

As a part of the program of synthetic work in the polynucleotide field which has been in progress in this Laboratory,[5] attention has been focused on the development of approaches to the specific synthesis of the naturally occurring $C_{3'}$–$C_{5'}$ inter-ribonucleotidic linkage.[2] The presence of the 2′-hydroxyl group in ribonucleosides presents a serious complication in synthetic work and the underlying aim has been to prepare ribonucleoside-3′ phosphate derivatives[6] in which the 2′-hydroxyl group is protected by a group that can be removed subsequently under adequately mild conditions. The key intermediates used in the previously described approach[2] were the 2′-*O*-tetrahydropyranylribonucleoside-3′ phosphates. The condensation of these intermediates with protected ribonucleosides bearing free 5′-hydroxyl groups followed by a work-up inclusive of an acidic treatment to remove the tetrahydropyranyl group afforded several $C_{3'}$–$C_{5'}$-linked ribodinucleotides.[2] During this work it wasobs erved that the rate of removal of the tetrahydropyranyl group varied considerably in different mononucleotide derivatives[7] and, in particular, that this group was consistently more stable in the synthetic products containing the phosphodiester linkage than in the mononucleotides.[2a,8] Often the acidic conditions necessary for the complete removal of the tetrahydropyranyl group from the synthesized dinucleotides caused detectable isomerization of the inter-ribonucleotidic linkage ($C_{3'}$–$C_{5'}$ ⇄ $C_{2'}$–$C_{5'}$) and, therefore, caution was necessary in the duration of the acidic treatment. This drawback in the use of the tetrahydropyranyl group was anticipated to be more serious in the isolation of uridine oligonucleotides containing exclusively the $C_{3'}$–$C_{5'}$ inter-ribonucleotidic linkages after polymerization of the previously prepared 2′-*O*-tetrahydropyranyluridine-3′ phosphate.[2a] An alternative approach, which has now been developed, aims at the use of an alkali-labile group for the protection of the 2′-hydroxy group in ribonucleoside-3′ phosphates. The particular merit in the use of an alkali-labile group was inferred from the previous observations[2a,9] which showed that while under acidic conditions some isomerization ($C_{3'}$–$C_{5'}$ ⇄ $C_{2'}$–$C_{5'}$) of the inter-ribonucleotidic linkage accompanies the hydrolytic reaction, under alkaline conditions no such isomerization can be detected.[10] The present communication reports on relatively simple methods for the preparation of 2′,5′-di-*O*-acetyluridine-3′ phosphate and 2′-*O*-acetyluridine-3′ phosphate and on the successful use of these intermediates in the specific synthesis of $C_{3'}$–$C_{5'}$-linked uridine oligonucleotides. Preliminary reports on parts of this work have already appeared.[11,12]

(1) Paper XVIII: R. K. Ralph, W. J. Connors, H. Schaller and H. G. Khorana, *J. Am. Chem. Soc.*, **85**, 1983 (1963).

(2) Previous papers in this series which deal with the present topic: (a) paper XIV, M. Smith, D. H. Rammler, I. H. Goldberg and H. G. Khorana, *ibid.*, **84**, 430 (1962); (b) paper XVI, D. H. Rammler and H. G. Khorana, *ibid.*, **84**, 3112 (1962).

(3) This work has been supported by grants from the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service, and the National Science Foundation, Washington, D. C.

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(5) H. G. Khorana, "Some Recent Developments in the Chemistry of Phosphate Esters of Biological Interest," John Wiley and Sons, Inc., New York, N. Y., 1961.

(6) In principle, the specific synthesis of the $C_{3'}$–$C_{5'}$ inter-ribonucleotide bond may be accomplished either by the condensation of a suitably protected ribonucleoside-5′ phosphate with a second ribonucleoside bearing a free 3′-hydroxyl function or by the condensation of a suitably protected ribonucleoside-3′ phosphate with a ribonucleoside bearing a free 5′-hydroxyl group. A systematic investigation[2b] showed the latter approach to be the more practical.

(7) Observations bearing on this point have been recorded in previous papers of this series. For example, the rate of hydrolysis of the tetrahydropyranyl group in 3′-*O*-tetrahydropyranyldeoxycytidine-5′ phosphate was slower than that in the corresponding N^6-acetyl or benzoyl derivative]footnote 50 in H. G. Khorana, A. F. Turner and J. P. Vizsolyi, *J. Am. Chem. Soc.*, **83**, 686 (1961)]. The tetrahydropyranyl group in 2′-*O*-tetrahydropyranyluridine-3′ phosphate is twice as labile as the corresponding group in 2′-*O*-tetrahydropyranyluridine-5′ phosphate (footnote 32 in ref. 2a). There is significant difference in the rate of hydrolysis of the 2′-*O*- and 5′-*O*-tetrahydropyranyl groups in 2′,5′-di-*O*-tetrahydropyranyluridine-3′ phosphate (present work).

(8) Compare the conditions used for the removal of tetrahydropyranyl groups in mononucleotides[7] with the conditions necessary for removal of the same group in uridylyl-(3′ → 5′)-uridine and uridylyl-(3′ → 5′)-adenosine derivatives.[2]

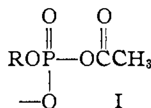
(9) D. M. Brown, D. I. Magrath, A. H. Neilson and A. R. Todd, *Nature*, **177**, 1124 (1956).

(10) The lack of migration in the inter-ribonucleotidic linkage on ammoniacal and sodium hydroxide treatment was confirmed; present work and unpublished experiments of Dr. P. T. Gilham in this Laboratory.

(11) (a) D. H. Rammler and H. G. Khorana, *Biochem. Biophys. Res. Comm.*, **7**, No. 2, 147 (1962); (b) *ibid.*, **8**, 61 (1962).

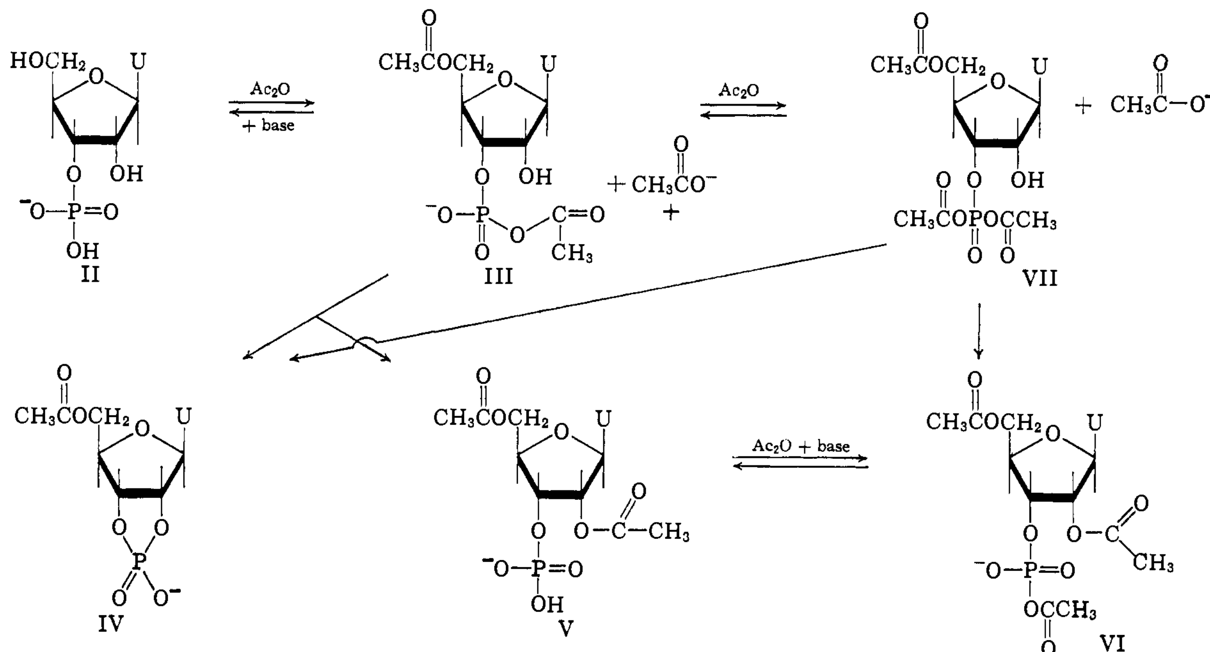
(12) Y. Lapidot and H. G. Khorana, *Chem. Ind.* (London), 166 (1963).

2′,5′-Di-*O*-acetyluridine-3′ Phosphate.—The reaction of an acylating agent such as acetic anhydride with the anion of a phosphomonoester (such as a mononucleotide) rapidly gives an acyl phosphate[13] (I). In the case of ribonucleoside-3′ phosphates (II) the corresponding mixed anhydride III[14] could give the 2′,3′-cyclic phosphate[5] IV or, alternatively, it could



cause acetylation of the neighboring 2′-hydroxyl group to give V. (The latter would then again form rapidly the mixed anhydride VI.)

Chart I. Preparation of 2′,5′-Di-*O*-acetyluridine-3′ Phosphate



Compound III could react further with acetic anhydride to form VII, although the amount of VII could not be expected to be large. Nevertheless, VII, which would be extremely reactive, could provide a route to the cyclic phosphate IV and the 2′-*O*-acetyl derivative VI. Acetylation of the 2′-hydroxyl group would probably also occur by direct attack of the acylating agent on III or even VII.

With the above theoretical considerations, a variety of conditions was examined for the acetylation of uridine-3′ phosphate (II). Acetylation with an excess of acetic anhydride under mildly acidic conditions (acetic acid, trifluoroacetic acid) or in aqueous sodium acetate buffer mainly caused 2′,3′-cyclic phosphate formation. Reaction in dry pyridine with an excess of acetic anhydride gave a substantial amount of 2′,5′-di-*O*-acetyl derivative V, it being isolated pure in about 35% yield. The use of an excess of acetyl bromide in pyridine gave an increased yield (51%) of V.

In further experiments, the inclusion of some water in the pyridine–acetic anhydride mixture influenced the reaction markedly in favor of the 2′-*O*-acetyl derivative V and, by using a very large excess of acetic anhydride, the yield of the latter could be increased to 85–90%. A possible function of water in these experiments appeared to be that of furnishing acetate ions and, therefore, acetylation was next tried in an anhydrous medium in the presence of tetraethylammonium acetate[15] as a source of acetate ions. The yield of the 2′-*O*-acetyl derivative IV was, indeed, high (92%) under these conditions. From these experiments it seemed probable that the undesired cyclic phosphate IV arose mainly *via* the diacetyl phosphate intermediate[16] VII and that the acetate ions inhibited its formation.

Attention was next directed to the relative concentration of the catalytic base and acetic anhydride since this factor would clearly influence the rate of acetylation of the 2′-hydroxyl group as well as the concentration of VII. The reaction of anhydrous pyridinium uridine-3′ phosphate with a tenfold excess of acetic anhydride in the presence of an equivalent amount of tetraethylammonium acetate with or without added inert solvent gave the desired 2′,5′-di-*O*-acetyluridine-3′ phosphate quantitatively. It is significant that the addition of pyridine in an amount stoichiometric with acetic anhydride caused the formation of a slight amount (1–2%) of the cyclic phosphate IV.

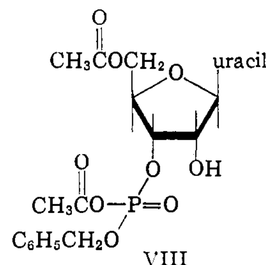
2′,5′-Di-*O*-acetyluridine-3′ phosphate (V) as prepared above was characterized by its lack of reaction with dicyclohexylcarbodiimide (DCC) under conditions which would have caused quantitative conversion to

(13) A. W. D. Avison, *J. Chem. Soc.*, 732 (1952); H. G. Khorana and J. P. Vizsolyi, *J. Am. Chem. Soc.*, **81**, 4660 (1959).

(14) The acetylation of the 5′-hydroxyl group will occur in the normal fashion.

(15) The base tetraethylammonium hydroxide has previously been used in this Laboratory for solubilization of oligonucleotides; H. Schaller, G. Weimann and H. G. Khorana, *J. Am. Chem. Soc.*, in press.

(16) The conclusion that the monoacetyl (III) could react further to the trisubstituted derivative VII is supported by experiments with uridine-2′-(or 3′) benzyl phosphate. Acetylation with acetic anhydride-pyridine mixture under anhydrous conditions caused the formation of some 5′-*O*-acetyluridine-2,′3′ cyclic phosphate (10%) (see Experimental). Since uridine-2′(or 3′) benzyl phosphate itself is stable in pyridine over prolonged periods, the cyclic phosphate could have arisen only by the further activation of the benzyl phosphate to form the mixed anhydride VIII.

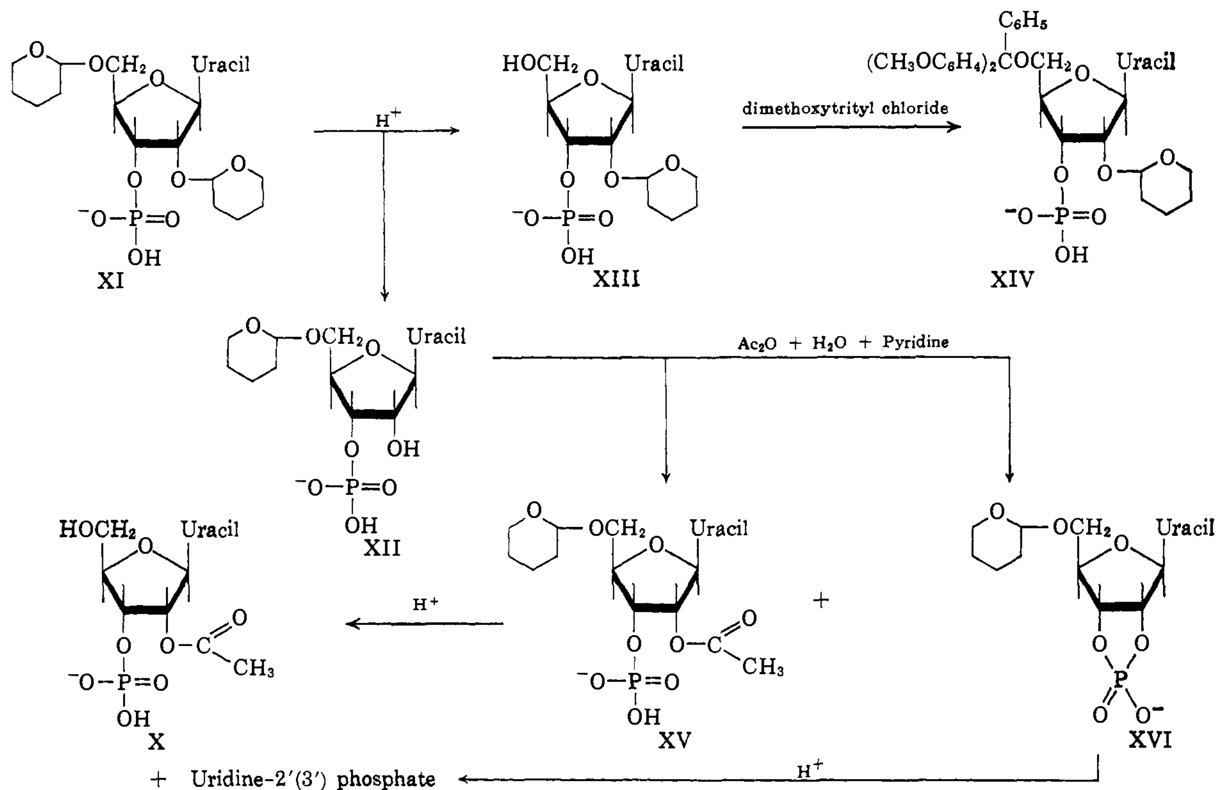


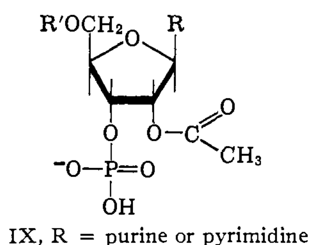
the 2′,3′-cyclic phosphate if the 2′-hydroxyl group had been free. In another experiment, this derivative was treated with DCC in the presence of an excess of methyl alcohol. Conversion to the monomethyl ester was quantitative and subsequent treatment with ammonia afforded methyl uridine-3′ phosphate. This ester was completely degraded by pancreatic ribonuclease to uridine-3′ phosphate. The result of this model experiment thus was encouraging for further work with the above approach in the synthesis of $C_{3'}$–$C_{5'}$ inter-ribonucleotidic linkage. Uridylyl-(3′ → 5′)-adenosine was, in fact, obtained in 84% yield by the condensation of 2′,5′-di-*O*-acetyluridine-3′ phosphate with N,N,$O^{2'}$,$O^{3'}$-tetrabenzoyladenosine (two molar equivalents) in the presence of DCC followed by an ammoniacal treatment to remove all of the protecting groups.

2′-*O*-Acetyluridine-3′ Phosphate.—The application of the above approach to the synthesis of $C_{3'}$–$C_{5'}$-linked ribo-oligonucleotides by polymerization of mononucleotides would require 2′-*O*-acylribonucleoside-3′ phosphates, while the synthesis by stepwise procedures would require intermediate of the type IX, in which the group R′ can be selectively removed. In the present work, 2′-acetyluridine-3′ phosphate was prepared and used in the polymerization reaction described below.

Two methods were developed for the synthesis of 2′-*O*-acetyluridine-3′ phosphate (X). The starting material in the first method was 5′-*O*-tetrahydropyranyl-uridine-3′ phosphate (XII), which in turn was prepared from 2′,5′-di-*O*-tetrahydropyranyluridine-3′ phosphate (XI). The preparation of the latter in essentially quantitative yield from uridine-3′ phosphate was described earlier.[2b] Carefully controlled acidic treatment of XI gave, as the main product, a mixture of the isomeric monotetrahydropyranyl derivatives XII and XIII in which the 5′-*O*-tetrahydropyranyl derivative XII predominated (75%).[17] For separation of the desired XII, the mixture of XII and XIII was treated with di-*p*-methoxytrityl chloride[2b] in dry pyridine so as to convert XIII to the readily separable di-*p*-methoxytrityl derivative XIV.[18] Although, under the conditions used, some reaction also occurred with XII, the major portion of the latter was isolated pure by subsequent partition chromatography. The compound was obtained in a crystalline form as its triethylammonium salt. The reaction of this product with acetic anhydride in aqueous pyridine gave, presumably, a mixture of 2′-*O*-acetyl-5′-*O*-tetrahydropyranyluridine-3′ phosphate (XV) and 5′-*O*-tetrahydropyranyluridine-2′,3′-cyclic phosphate (XVI) (Chart II).[19] The mixture was kept under acidic conditions to give, in turn, a mixture of uridine-2′ (or 3′) phosphate and 2′-*O*-acetyluridine-3′ phosphate (X) from which the latter

CHART II. PREPARATION OF 2′-*O*-ACETYLURIDINE-3′ PHOSPHATE





IX, R = purine or pyrimidine

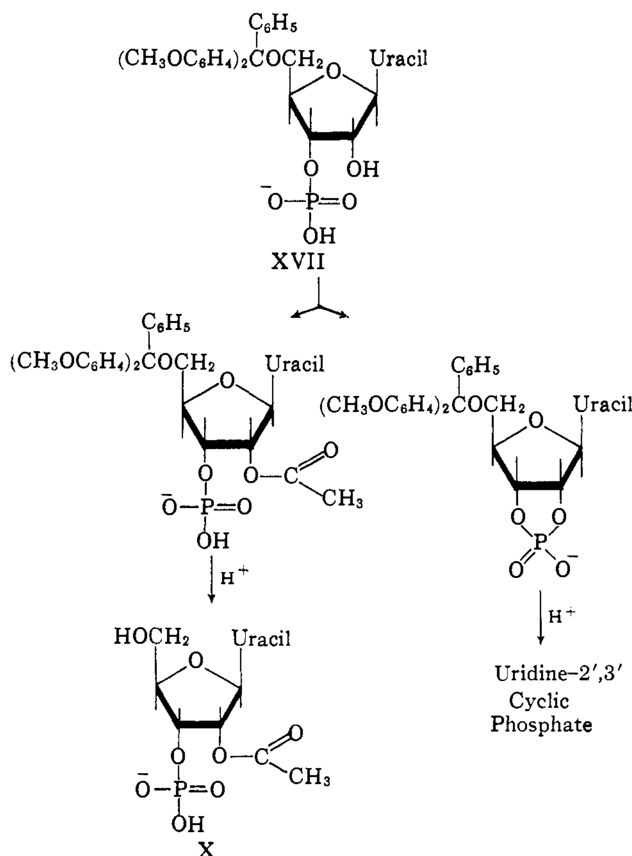
(17) The ratio of the two isomers was determined by selective conversion of XII to 5′-*O*-tetrahydropyranyluridine-2′,3′ cyclic phosphate (XVI) and then to the corresponding N-phosphoryl-N,N,-dicyclohexylureas on treatment with DCC in aqueous pyridine [C. A. Dekker and H. G. Khorana, *J. Am. Chem. Soc.*, **76**, 3522 (1954); H. G. Khorana, G. M. Tener, R. S. Wright and J. G. Moffatt, *ibid.*, **79**, 430 (1957)]. It should be noted that under the conditions (20% aqueous pyridine at room temp.) used, only the formation of the five-membered 2′,3′-cyclic phosphate occurs. The reaction of 2′-*O*-tetrahydropyranyluridine-3′ phosphate to form the corresponding pyrophosphate or the 3′,5′-cyclic phosphate [M. Smith, G. I. Drummond and H. G. Khorana, *ibid.*, **83**, 693 (1961)] is completely inhibited by the water present.

(18) *Cf.* the separation of 2′-*O*-tetrahydropyranyluridine-5′ phosphate from 2′-*O*-tetrahydropyranyluridine-3′ phosphate by selective reaction of the latter with trityl chloride.[2a]

(19) This work was completed before the conditions for the complete avoidance of the cyclic phosphate formation were discovered.

was isolated pure by partition chromatography on a cellulose column.

In the second route to 2′-*O*-acetyluridine-3′ phosphate, uridine-3′ phosphate was converted to 5′-*O*-di-*p*-methoxytrityluridine-3′ phosphate (XVII)[20] by direct reaction under controlled conditions with di-*p*-methoxytrityl chloride in pyridine. The major product was the desired one, although some of the bis-di-*p*-methoxytrityl derivative also was formed. The reaction of 5′-*O*-di-*p*-methoxytrityluridine-3′ phosphate (XVII) with acetic anhydride in aqueous pyridine gave again a

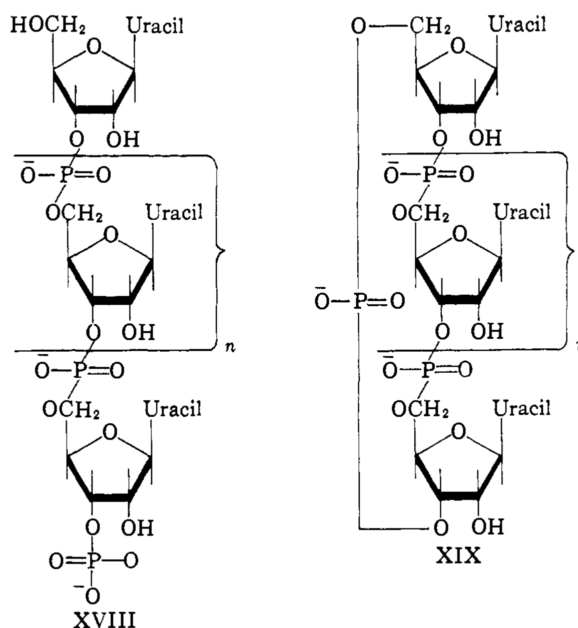


mixture of the 2′,3′-cyclic phosphate[19] and the 2′-*O*-acetyl phosphate. After very brief acidic treatment to remove the di-*p*-methoxytrityl group, the products were separated by partition chromatography on a cellulose column.

2′-*O*-Acetyluridine-3′ phosphate as prepared by either of the two methods described was characterized by preparation from it of methyl uridine-3′ phosphate which was completely degraded by pancreatic ribonuclease. In another experiment, it was treated with DCC in dry pyridine in the presence of 2′,3′-di-*O*-benzoyluridine. Although a large amount of uridine-2′ (or 3′) phosphate was recovered and the major new product obtained was uridine-3′,5′ cyclic phosphate,[21] uridylyl-(3′ → 5′)-uridine (6%) obtained was completely degraded by pancreatic ribonuclease to uridine-3′ phosphate and uridine.

The Synthesis of Uridine Oligonucleotides.—2′-*O*-Acetyluridine-3′ phosphate was allowed to react in dry pyridine with dicyclohexylcarbodiimide under the standard conditions developed previously for polymerization of mononucleotides.[22] After subsequent removal of the acetyl groups by an ammoniacal treatment, the products were separated by chromatography on a DEAE–cellulose column. The pattern of elution was similar to that described previously for thymidine-5′ phosphate[22] or thymidine-3′ phosphate[23] except that the polymerization did not go as far.[24] The products were further purified by a combination of paper chromatography and paper electrophoresis. As before, the products could be classified into two major groups, the linear oligonucleotides (general structure XVIII) and the cyclic oligonucleotides (general structure XIX) and, in addition, side products (see below) were present.

Uridine-3′ phosphate, uridine dinucleotide, uridylyl-(3′ → 5′)-uridine-3′ phosphate, and the corresponding trinucleotide XVIII (n = 1) were characterized by their position of elution from the column and their mobilities on paper chromatograms and paper electro-



phoresis (Tables I and II). The dinucleotide XVIII (n = 0) gave, after incubation with alkaline bacteria-phosphomonoesterase, uridylyl-(3′ → 5′)-uridine which, in turn, was completely degraded by pancreatic ribonuclease to equal amounts of uridine-3′ phosphate and uridine. The trinucleotide, uridylyl-(3′ → 5′)-uridylyl-(3′ → 5′)-uridine-3′ phosphate, was characterized analogously, the degradation with pancreatic ribonuclease, again proceeding to completion. Smaller amounts of higher oligonucleotides were evidently formed in the polymerization reactions, but their purification was not attempted.

Of the cyclic series of compounds, the smallest member was uridine-3′,5′ cyclic phosphate.[25] This was

(20) The reaction of adenosine-3′ phosphate with di-*p*-methoxytrityl chloride under similar conditions gave 5′-*O*-di-*p*-methoxytrityladenosine-3′ phosphate as the only monosubstituted product. That no migration of the phosphoryl group occurred was shown by the removal of the di-*p*-methoxytrityl group and subsequent chromatography in solvent systems which separate adenosine-2′ and -3′ phosphate.

(21) In an unpublished experiment by Dr. M. Smith in this Laboratory, the reaction of triethylammonium 2′-*O*-tetrahydropyranyluridine-3′ phosphate with DCC in the presence of an excess of *p*-nitrophenol also gave uridine-3′,5′ cyclic phosphate as the major product and not the nitrophenyl ester. Under identical conditions a nucleoside-5′ phosphate gave only the nitrophenyl ester [experiment by Dr. J. G. Moffatt quoted in W. E. Razzell and H. G. Khorana, *J. Biol. Chem.*, **234**, 2105 (1959)]. There is thus a great tendency for the 5′-hydroxyl group to participate when the 3′-phosphate group is activated.

(22) H. G. Khorana and J. P. Vizsolyi, *J. Am. Chem. Soc.*, **83**, 675 (1961); H. G. Khorana, A. F. Turner and J. P. Vizsolyi, *ibid.*, **83**, 686 (1961); H. G. Khorana, J. P. Vizsolyi and R. K. Ralph, *ibid.*, **84**, 414 (1962); R. K. Ralph and H. G. Khorana, *ibid.*, **83**, 2926 (1961).

(23) A. F. Turner and H. G. Khorana, *ibid.*, **81**, 4651 (1959).

(24) This is ascribed to the fact that the protected starting material has so far been available in rather small quantities and the polymerization was carried out with only 0.0625 mmole. Larger scale reactions (see ref. 22) have given better results.

(25) M. Smith, G. I. Drummond and H. G. Khorana, *J. Am. Chem. Soc.*, **83**, 698 (1961).

clearly distinguishable from uridine-2′,3′-cyclic phosphate by its greater stability to acid and alkali. The absence of the 2′,3′-cyclic phosphate isomer further proves that the 2′-hydroxyl group was effectively blocked throughout the course of polymerization reaction. Uridine-3′,5′-cyclic phosphate was present in rather large amount (see Experimental) because of the rather high tendency of the 5′-hydroxyl group to participate in the intramolecular reaction.[21,26]

The cyclic dinucleotide and the corresponding trinucleotide were characterized (R_f's in Tables I and II) by resistance to phosphomonoesterase and by the ratio of their paper electrophoretic mobilities to those of linear members at acid and neutral *p*H. It is of particular interest that the cyclic dinucleotide *was* degraded by pancreatic ribonuclease to uridine-3′ phosphate, although the rate appeared to be slower as compared with uridylyl-(3′ → 5′)-uridine-3′ phosphate. Similarly, the rate of alkaline hydrolysis was slower than that of uridylyl-(3′ → 5′)-uridine. While the latter was completely degraded in 6 hr. (in 0.5 *N* sodium hydroxide) at room temperature, the cyclic dinucleotide was degraded to the extent of 15–20% in 6 hr. and about 70% in 54 hr. These results indicate that in the cyclic dinucleotide the 2′-hydroxyl group is able to participate in the degradative reactions, although this participation is somewhat less effective than that in the regular inter-ribonucleotidic linkage.

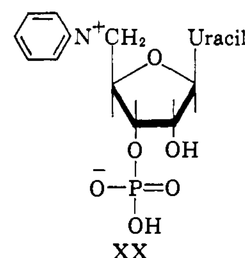
TABLE I
PAPER CHROMATOGRAPHY OF DIFFERENT COMPOUNDS

Compound	R_f Solvent A	R_f Solvent B	R_f Solvent C
Uridine-3′ phosphate	0.085	0.3	0.34
Uridine-2′,3′-cyclic phosphate	.3	.58	.50
Uridine-3′,5′-cyclic phosphate	.28	.58	.49
5′-*O*-Acetyluridine-3′ phosphate	...	.42	...
5′-*O*-Acetyluridine-2′,3′-cyclic phosphate	...	.69	...
2′,5′-Di-*O*-acetyluridine-3′ phosphate	...	.55	...
5′-*O*-Di-*p*-methoxytrityluridine-3′ phosphate	0.46	...	...
Uridine cyclic dinucleotide	...	0.6	0.37
Uridine cyclic trinucleotide	...	...	.25
Uridine cyclic tetranucleotide	...	...	.16
Uridylyl-(3′ → 5′)-uridine	0.16	...	.37
Uridylyl-(3′ → 5′)-uridine-3′ phosphate	...	0.51	.24
Uridylyl-(3′ → 5′)-uridylyl-(3′ → 5′)-uridine-3′ phosphate	...	...	.15
Uridylyl-(3′ → 5′)-uridylyl-(3′ → 5′)-uridine	...	...	.28

TABLE II
PAPER ELECTROPHORETIC MOBILITIES RELATIVE TO THAT OF URIDINE-3′ PHOSPHATE

	*p*H 7.1	*p*H 2.7
5′-(N-Pyridinium)-uridine-3′ phosphate	0.36	0
Uridine-3′,5′ cyclic phosphate	.63	1
Uridine cyclic dinucleotide	.78	1.13
Uridylyl-(3′ → 5′)-uridine-3′ phosphate (UpUp)	1.05	1.13
Uridine cyclic trinucleotide	0.91	1.21
Uridylyl-(3′ → 5′)-uridylyl-(3′ → 5′)-uridine-3′ phosphate	1.04	1.2
2′,5′-Di-*O*-acetyluridine-3′ phosphate	0.87	...

Of the minor products, a prominent one was that which showed ultraviolet absorption properties additive of uridine and pyridinium chromophores. The compound is probably XX. Compounds of this type have been encountered previously in the deoxyribo-oligonucleotide work[22,23] and the mechanism of their formation has been established.[27]



Concluding Remarks.—Previous chemical methods for polymerization of ribonucleotides have yielded oligonucleotides containing random $C_{2'}$–$C_{5'}$ and $C_{3'}$–$C_{5'}$ internucleotide bonds.[28–30] The development now reported constitutes the first specific synthesis of ribo-oligonucleotides containing $C_{3'}$–$C_{5'}$ internucleotidic linkages. The extent of polymerization realized so far is not very satisfactory, however, and further work on this method is, therefore, necessary. The principles involved, however, are now clear for the elaboration of ribopolynucleotide synthesis.

Experimental

General Methods.—Reagent grade pyridine was dried over calcium hydride for several weeks. All evaporations were carried out using a rotary evaporator under reduced pressure. For condensation reactions involving DCC, solutions were rendered anhydrous by repeated evaporation of pyridine solutions using a high vacuum.

Paper chromatography was performed using the descending technique on double acid-washed Whatman No. 40 paper except where noted otherwise. The solvent systems used were: solvent A, isopropyl alcohol–concd. ammonia–water (7:1:2, v./v.); solvent B, ethyl alcohol–1 *M* ammonium acetate, *p*H 6.5, (5:2, v./v.); solvent C, *n*-propyl alcohol–concd. ammonia–water (55:10:35, v./v.); solvent D, *n*-butyl alcohol–acetic acid–water (5:2:3, v./v.); R_f's of different compounds are given in Table I and sometimes in text.

Paper electrophoresis was performed in an apparatus similar to that of Markham and Smith[31] or in a commercially available apparatus designed on the same principle and capable of giving a potential of 5000 volts. The buffers used were: phosphate buffer, *p*H 7.1, 0.03 *M*; triethylammonium bicarbonate, *p*H 7.5, 0.05 *M*; citrate buffer, *p*H 2.7, 0.03 *M*; and ammonium formate buffer, *p*H 3.5, 0.05 *M*. Electrophoretic mobilities are given in Table II.

Degradation of uridine-3′ methyl phosphate, ribodinucleotides and other oligonucleotides by pancreatic ribonuclease was performed as follows: The incubation mixture consisted of about 0.5–1 μmole of the substrate, crystalline pancreatic ribonuclease (50 μg.) and tris-hydroxymethylaminomethane buffer (*p*H 7.5, 5 μmoles) in a total volume of 0.1 ml. The incubation was carried out for 2–3 hr. at 37° and the products were determined either by paper chromatography in solvent A or C or by paper electrophoresis at *p*H 7.1.

Dephosphorylation of terminal phosphomonoester groups was carried out by incubation of the oligonucleotides with *E. coli* alkaline phosphomonoesterase[32] using a preparation generously given by Dr. J. Schwartz of the Rockefeller Institute. The standard conditions were: 2–10 optical density units (260 mμ) of the substrate as lyophilized powder in 0.05–0.1 ml. of tris-hydroxymethylaminomethane buffer (*p*H 8, 0.1 *M*) and 0.005 ml. of the enzyme preparation (about 2 mg. protein/ml.) were incubated at 37° for 3 hr. The products were then directly chromatographed in solvent A or C.

The Reaction of Uridine-3′ Phosphate with Acetic Anhydride in Trifluoroacetic Acid.—A mixture of disodium uridine-3′ phos-

(26) Using a high concentration of protected ribonucleoside-3′ phosphates it is hoped that the intramolecular cyclization reaction can be inhibited. Even in the published work on thymidine-3′ phosphate polymerization,[23] thymidine-3′,5′ cyclic phosphate formation was not overwhelming.

(27) G. Weimann and H. G. Khorana, *J. Am. Chem. Soc.*, **84**, 419 (1962).
(28) M. Smith, J. G. Moffatt and H. G. Khorana, *ibid.*, **80**, 6204 (1958).
(29) A. M. Michelson, *J. Chem. Soc.*, 1371 (1959).
(30) G. Schramm, H. Grotsch and W. Pollmann, *Angew. Chem.*, **74**, 53 (1962).
(31) R. Markham and J. D. Smith, *Biochem. J.*, **52**, 552 (1952).
(32) A. Garen and C. Levinthal, *Biochim. Biophys. Acta*, **38**, 470 (1960).

phate[33] (0.05 g.), acetic anhydride (2 ml.) and trifluoroacetic acid (0.1 ml.) was shaken mechanically for 3 hr. Chromatography in solvent B showed no starting material. The products, by a combination of paper chromatography and paper electrophoresis, were shown to be uridine-2',3' cyclic phosphate and 5'-*O*-acetyluridine-2',3' cyclic phosphate. During work-up considerable breakdown of these products to, respectively, 5'-*O*-acetyluridine-2'(3') phosphate and uridine-2'(3') phosphate occurred.

The Reaction of Uridine-3' Phosphate with Acetic Anhydride in Aqueous Sodium Acetate.—A solution of uridine-3' phosphate (0.025 g. of disodium salt) in 2 ml. of saturated aqueous sodium acetate and 1 ml. of water was treated under agitation with acetic anhydride (0.5 ml.). Examination of products after 1 hr. by a combination of paper chromatography (solvent B) and paper electrophoresis showed the major product to be uridine-2',3' cyclic phosphate, the other products being 5'-*O*-acetyluridine-2',3' cyclic phosphate and a small amount of 2',5'-di-*O*-acetyluridine-3' phosphate.

2',5'-Di-*O*-acetyluridine-3' Phosphate. Using Acetic Anhydride in Dry Pyridine.—Acetic anhydride (1 ml., 10 mmoles) was added to an anhydrous pyridine solution (2 ml.) of uridine-3' phosphate (0.1 g. of free acid, 0.32 mmoles) and the sealed reaction mixture was kept for 2 hr. at room temp. Methyl alcohol (2 ml.) was then added and the reaction mixture then directly chromatographed in solvent B. Two ultraviolet absorbing spots[34] containing nucleotidic material were detected. The faster spot (62%, R_f 0.66 in solvent B) corresponded to 5'-*O*-acetyluridine-2',3' cyclic phosphate and the slower spot (38%, R_f 0.54 in solvent B) corresponded to 2',5'-di-*O*-acetyluridine-3' phosphate.

The ultraviolet absorption characteristics of 5'-*O*-acetyluridine-2',3' cyclic phosphate in water were: λ_{max} 258.5 and 207 mμ; λ_{min} 228 mμ. Its mobility on paper electrophoresis at *p*H 7.1 was 0.57 with respect to uridine-3' phosphate. On treatment with 80% acetic acid at room temp. for 12 hr., it gave uridine-2'(3') phosphate and 5'-*O*-acetyluridine-2'(3') phosphate.

2',5'-Di-*O*-acetyluridine-3' phosphate had the properties: λ_{max} in H_2O, 260, 207 mμ; λ_{min} 228 mμ. Its mobility on paper electrophoresis at *p*H 7.1 was 0.87 relative to uridine-3' phosphate.

An anhydrous pyridine solution was kept at room temperature for 5 days. Examination of an aliquot by paper chromatography in solvent B showed only unchanged starting material.

The absence of free 2'-hydroxyl group in 2',5'-di-*O*-acetyluridine-3' phosphate was further shown as follows: Pyridinium 2',5'-di-*O*-acetyluridine-3' phosphate (2.87 μmoles) was dissolved in a mixture of pyridine (1 ml.) and water (0.2 ml.) and the solution treated with DCC (0.150 g.) at room temp. Paper chromatography of aliquots removed at 3.5 hr. and 6.5 hr. in solvent B showed only unchanged starting material.

A sample (3.7 μmoles) of 2',5'-di-*O*-acetyluridine-3' phosphate (free acid, lyophilized powder) was kept at room temp. in 0.5 ml. of 80% acetic acid. Aliquots were removed after 30, 60 and 90 min. and examined by paper chromatography in solvent B. No breakdown of the starting material could be detected.

Using Acetyl Bromide in Dry Pyridine.—To a pyridine solution (1 ml.) of uridine-3' phosphate (0.06 g., 0.186 mmole of free acid) was added acetyl bromide (0.2 ml.) and the mixture containing the precipitate, which formed, was shaken for 1 hr. at room temp. Methyl alcohol was added to the resulting reddish mixture and the solution evaporated twice with readdition of methyl alcohol. The residue was dissolved in 10 ml. of 1 *M* sodium acetate (*p*H 5) solution and acid-washed Norit A charcoal (1 g.) was added. After thorough stirring the charcoal was centrifuged down and after removal of the supernatant washed three times with water. The nucleotidic material was then eluted from charcoal with 50% aqueous pyridine, the charcoal being removed by filtration on a Celite pad. The recovery of the nucleotidic material was 0.155 mmole, as determined spectrophotometrically after removal of pyridine. Paper chromatography in solvent B showed the products to be 5'-*O*-acetyluridine-2',3' cyclic phosphate and 2',5'-di-*O*-acetyluridine-3' phosphate (58%).

Acetylation of Uridine-3' Phosphate with Acetic Anhydride in Aqueous Pyridine.—Disodium uridine-3' phosphate (0.015 g.) was dissolved in 0.3 ml. of water. To the solution was added rapidly under agitation a previously prepared mixture of pyridine (1 ml.) and acetic anhydride (1 ml.). The clear solution was directly chromatographed in solvent B. The faster band of 5'-*O*-acetyluridine-2',3' cyclic phosphate was present in the amount of 24%, while the major product (76%) was 2',5'-di-*O*-acetyluridine-3' phosphate. The identity of the latter product was further checked by elution of the band from the paper chromatogram, removal of salt by treatment with pyridinium Dowex-50 resin, lyophilization and subsequent paper electrophoresis at *p*H 7.1. Furthermore, ammoniacal treatment of the product gave uridine-3' phosphate, as identified by paper chromatography in solvent A. 5'-*O*-Acetyluridine-2',3'-cyclic phosphate after similar treatment gave uridine-2',3' cyclic phosphate.

In the above experiment when the amount of pyridine and acetic anhydride was increased to 2.5 ml. each the yield of 2',5'-di-*O*-acetyluridine-3' phosphate was higher (85–90%).

Acetylation of Uridine-3' Phosphate in the Presence of Tetraethylammonium Acetate. (a) **In Dry Pyridine.**—A mixture of pyridinium uridine-3' phosphate (0.1 mmole) and tetraethylammonium acetate (1 mmole, 1 ml. solution of an aqueous 1 *M* solution) was rendered anhydrous by repeated evaporation of pyridine. Finally dry pyridine (0.5 ml.) and then acetic anhydride (0.1 ml., 1 mmole) were added and the sealed reaction mixture kept at room temp. for 2 hr. Water (0.5 ml.) was added and the reaction mixture kept further at room temp. for 4–5 hr. Paper electrophoresis (*p*H 7.1) showed the complete hydrolysis of the acetyl-phosphate mixed anhydride by this time. Analysis by paper chromatography and electrophoresis showed only a small amount of 5'-*O*-acetyluridine-2',3' cyclic phosphate (about 5%), the major product being 2',5'-di-*O*-acetyluridine-3' phosphate.

(b) **Without the Addition of Pyridine.**—Pyridinium uridine-3' phosphate (0.1 mmole) and tetraethylammonium acetate (1 mmole) were rendered anhydrous by repeated evaporation of pyridine. During the last evaporation the suction under vacuum was continued until a hard gum remained. Acetic anhydride (1 mmole) was added and the sealed reaction mixture kept for 2 hr. at room temp. Water (0.5 ml.) and pyridine (0.5 ml.) were added and after a further 4–5 hr. at room temp. the solution was evaporated. The residue was redissolved in water and the solution passed in the cold through a column (10 cm. × 1 cm.) of Dowex-50 (H^+) ion exchange resin. The total acidic effluent was lyophilized. The sole product thus obtained was 2',5'-di-*O*-acetyluridine-3' phosphate.

Acetylation of Benzyl Uridine-2' (or 3') Phosphate.[35] (a) **With Acetic Anhydride in Dry Pyridine.**—Acetic anhydride (0.5 mmole) was added to a solution of benzyl uridine-2' (or 3') phosphate (0.02 mmole) in a dry pyridine (0.2 ml.) and the sealed mixture kept at room temp. for 1 hr. Water (0.2 ml.) was then added and after 1 hr. at room temp. the reaction mixture was directly chromatographed in solvent D. 2',5'-Di-*O*-acetyluridine-2' (or 3') benzyl phosphate 85% (R_f 0.61) was the major product. Other ultraviolet-absorbing products detected were: benzylpyridinium cation, R_f 0.72; 5'-*O*-acetyluridine-2',3' cyclic phosphate (10%, R_f 0.30); 2',5'-di-*O*-acetyluridine-2' (or 3') phosphate (5%, R_f 0.36).

(b) **With Acetic Anhydride in the Presence of Tetraethylammonium Acetate.**—A mixture of benzyl uridine-2' (or 3') phosphate (0.05 mmole) and tetraethylammonium acetate (1 mmole) was rendered anhydrous by repeated evaporation of pyridine. To the gum finally obtained was added acetic anhydride (1 mmole) and the reaction mixture was kept at room temp. for 2 hr. Water (0.5 ml.) and pyridine (0.5 ml.) were then added and after 1 hr. the solution was passed through a column (10 cm. × 1 cm.) of Dowex-50 (pyridinium form) ion exchange resin. The total effluent and aqueous pyridine washings were lyophilized. 2',5'-Di-*O*-acetyluridine-2' (or 3') benzyl phosphate was the sole product; R_f in solvent B, 0.85, the starting material has R_f 0.78. The product had an electrophoretic mobility (*p*H 7.1) of 0.81 relative to the starting material.

Acidic Hydrolysis of Tetrahydropyranyl Derivatives of Nucleotides.—Solutions were prepared in 80% acetic acid of various nucleotide derivatives so as to give concentration of approximately 5 μmoles of nucleotide/ml. The solutions were kept at room temperature and aliquots removed at different intervals were chromatographed on Whatman No. 1 paper in solvent A. The nucleotide spots were eluted with water and their concentrations determined spectrophotometrically.

The half-lives of the tetrahydropyranyl groups in different compounds were found to be: 2'-*O*-tetrahydropyranyl group in 2',5'-di-*O*-tetrahydropyranyluridine-3' phosphate, 3 min.; 5'-*O*-tetrahydropyranyl group in 2',5'-di-*O*-tetrahydropyranyluridine-3' phosphate, 20 min.; 5'-*O*-tetrahydropyranyluridine-3' phosphate, 18 min.; 2'-*O*-tetrahydropyranyl group in 2',5'-di-*O*-tetrahydropyranyladenosine-3' phosphate, 8 min.; 5'-*O*-tetrahydropyranyl group in the same, 46 min.

Partial Hydrolysis of 2',5'-Di-*O*-tetrahydropyranyluridine-3' Phosphate. (a) **In 80% Acetic Acid.**—A solution of 2',5'-di-*O*-tetrahydropyranyluridine-3' phosphate[2b] (0.72 mmole) in 80% acetic acid (20 ml.) was kept at 0° for 4.5 hr. The solution was then evaporated at 5° to about 3 ml. and ice-cold water (150 ml.) was added. The total solution was lyophilized. The residue was dissolved in a small amount (10 ml.) of a mixture of isopropyl

(33) The purity of the sample used in all this work was carefully checked by chromatography on a Dowex-1 ion exchange resin column using the procedure described by W. E. Cohn and J. X. Kym, "Biochemical Preparations," Vol. V, D. Shemin, Ed., J. Wiley and Sons, Inc., New York, N. Y., 1957, p. 40.

(34) There is often present, as reported in earlier papers of this series, a fast ultraviolet-absorbing spot which displays λ_{max} at 295 mμ. It is non-nucleotidic and arises from the reaction of acetic anhydride with pyridine.

(35) Prepared by the method of G. M. Tener and H. G. Khorana, *J. Am. Chem. Soc.*, **77**, 3349 (1955).

alcohol–concd. ammonia–water (7:1:2) and the solution chromatographed on a Whatman No. 1 cellulose powder column as described previously for 2′,5′-di-*O*-tetrahydropyranyluridine-3′ phosphate.[2b] Fractions 222–236 contained the starting material (0.23 mmole, 30%); fractions 240–265 contained a mixture of 2′- and 5′-*O*-monotetrahydropyranyluridine-3′ phosphate (0.365 mmole, 50%) while fractions 269–272 contained uridine-3′ phosphate (0.098 mmole, 13.5%). The combined fractions were evaporated under reduced pressure and at low temp. in the presence of pyridine and the concentrated solutions stored in pyridine at 0°.

The mixture of 2′- and 5′-*O*-monotetrahydropyranyluridine-3′ phosphates was shown to consist of 25% of 2′-*O*-tetrahydropyranyluridine-3′ phosphate and 75% of 5′-*O*-tetrahydropyranyluridine-3′ phosphate as follows: A small amount (0.0025 mmole) of the ammonium salts of the above mixture was converted to the pyridinium salts by passage through a pyridinium Dowex-50 ion exchange resin column. The resulting pyridinium salts were taken up in 1 ml. of pyridine and water (0.2 ml.) and then DCC (0.1 g.) was added. Aliquots were removed from the reaction mixture at various times and chromatographed in solvent A. After 1 hour, two spots were detected, the slower corresponding in R_f to the starting material and the faster to, presumably, 5′-*O*-tetrahydropyranyluridine-2′,3′ cyclic phosphate. After 16 hr., three spots were detected. The slowest spot again corresponded in R_f to the starting material and did not change further in concentration even after 80 hr. of reaction time. The second spot was slowly converted to the fastest spot, presumably, corresponding to N-(5′-*O*-tetrahydropyranyluridine-2′ or 3′-phosphoryl)-N,N′-dicyclohexylurea.[17] After 80 hr., the only two spots present were those of 2′-*O*-tetrahydropyranyluridine-3′ phosphate (25%) and of the phosphorylurea.[17] The intermediate spot was shown to have only one negative charge when electrophorezed at *p*H 7.5. It gave after treatment with 80% acetic acid at room temp. for 20 hr. uridine-2′ (3′) phosphate.

(b) **With Dowex-50 (H^+) Resin.**—To an aqueous solution (15 ml.) of 2′,5′-di-*O*-tetrahydropyranyluridine-3′ phosphate (0.15 mmole), Dowex-50 (H^+) ion exchange resin was added in small portions with thorough shaking. The addition of the resin was discontinued when the *p*H fell to 2.4–2.5. The acidic solution was kept at room temp., aliquots were removed at different intervals and were examined by paper chromatography in solvent A. The yield of monotetrahydropyranyluridine-3′ phosphates was at a maximum (46%) after 20 min. These conditions were used for preparation of monotetrahydropyranyluridine-3′ phosphates which were isolated as described above under (a).

5′-*O*-Tetrahydropyranyluridine-3′ Phosphate.—An anhydrous pyridine (20 ml.) solution of the mixture of ammonium 2′- and 5′-*O*-monotetrahydropyranyluridine-3′ phosphate (0.81 mmole) as obtained above was treated at room temp. with 1.38 g. (4.06 mmoles) of di-*p*-methoxytrityl chloride.[2a] After 80 min.,[36] 100 ml. of 0.17 *M* ammonium hydroxide was added and the mixture extracted three times with 25-ml. portions of ether. The aqueous ammoniacal solution was applied to the top of a DEAE-cellulose (bicarbonate form) column (40 cm. × 4 cm. diameter). The column was washed with 500 ml. of 10% ethyl alcohol (*cf.* ref. 2a) followed by 500 ml. of water. Elution was begun with 2 l. of 0.1 *M* triethylammonium bicarbonate (*p*H 7.5). Eighteen-to twenty-ml. fractions were collected at a flow rate of about 1 ml./min. Fractions 39–73 contained pure 5′-*O*-tetrahydropyranyluridine-3′ phosphate (34.6%). Elution was then continued with 0.2 *M* triethylammonium bicarbonate (*p*H 7.5) in 20% ethyl alcohol (2 l.). The first peak eluted was a minor one and contained nucleotidic material which has not been identified. The second major peak corresponded to 5′-*O*-dimethoxytrityl-2′-*O*-tetrahydropyranyluridine-3′ phosphate and was contained in fractions 30–70 (renumbering of fractions after the first change of the eluent).

The pooled fractions containing 5′-*O*-tetrahydropyranyluridine-3′ phosphate were evaporated under reduced pressure in a vacuum. When most of triethylammonium bicarbonate was lost, the residual concentrated solution was treated with ethyl alcohol. Triethylammonium salt of the product crystallized as needles.

This product was completely pure by paper chromatography and by paper electrophoresis. That the product consisted exclusively of 5′-*O*-tetrahydropyranyluridine-3′ phosphate was shown as follows: A solution of the product (3.1 μmoles) in a mixture of pyridine (1 ml.), water (0.2 ml.) and triethylamine (0.01 ml.) was treated with DCC (0.150 g.). Examination by paper chromatography in solvent A after 12 hr. at room temperature showed the sole product to be 5′-*O*-tetrahydropyranyluridine-2′,3′ cyclic phosphate.

The major peak eluted above with 0.2 *M* triethylammonium bicarbonate in 20% ethyl alcohol contained, evidently, two di-*p*-methoxytrityl derivatives: one corresponded to 5′-*O*-dimethoxytrityl-2′-*O*-tetrahydropyranyluridine-3′ phosphate[2a] while the second was a much more labile derivative. The latter partially lost the dimethoxytrityl group on being kept in aqueous pyridine and was, presumably, 5′-*O*-tetrahydropyranyl-2′-*O*-dimethoxytrityluridine-3′ phosphate.[37]

Kinetic Study of the Di-*p*-methoxytritylation of Uridine-3′ Phosphate.—To a mixture of disodium uridine-3′ phosphate (0.015 g., 0.041 mmole) in dry pyridine (1 ml.) was added di-*p*-methoxytrityl chloride (0.068 g., 0.201 mmole). The sealed reaction mixture became homogeneous within a few minutes on shaking. Aliquots were removed at various time intervals and directly applied on paper chromatograms developed in solvent A. 5′-*O*-Di-*p*-methoxytrityluridine-3′ phosphate travelled faster (R_f about 0.4) than the starting nucleotide, whereas 2′,5′-bis-*O*-di-*p*-methoxytrityl derivative moved fastest (R_f 0.6–0.7) on paper chromatograms. The yields of products were determined spectrophotometrically after elution of spots with ammoniacal ethyl alcohol. The yield of 5′-*O*-di-*p*-methoxytrityluridine-3′ phosphate was: 65% in 20 min., about 80% in 50 min. This product slowly decreased and the ditrityl derivative increased in amount. While most of the starting material, uridine-3′ phosphate, had disappeared, a small amount (about 5%) persisted even after 390 min. time reaction.

In one experiment the above standard experiment was repeated and after 60 min. the reaction mixture was treated with 0.5 ml. of water. The aqueous pyridine solution was partly evaporated and then applied on Whatman 3 MM paper in solvent A. The main band (R_f 0.45) of 5′-*O*-di-*p*-methoxytrityluridine-3′ phosphate was cut out and eluted with 0.1 *M* ammonium hydroxide while still wet. The yield of pure product was 53%.

5′-*O*-Dimethoxytrityluridine-3′ Phosphate.—To a suspension of uridine-3′ phosphate (0.5 g., 1.36 mmoles, disodium salt) in dry pyridine (30 ml.) was added dimethoxytrityl chloride (2.33 g., 6.7 mmoles) and the mixture was shaken at room temperature for 2 hr., ammonium hydroxide (0.1 *M*, 10 ml.) was added and the solution extracted with ether (3 × 25 ml.) to remove dimethoxytritylcarbinol. The aqueous layer was evaporated to a small volume (10 ml.) and ethanol was added to clarify the cloudy solution. The *p*H was adjusted to about 9 and the solution was placed on a DEAE-cellulose (bicarbonate form) column (4.5 × 55 cm.). The column was washed with 10% ethanol–water solution (750–1000 ml.) and then eluted with a linear gradient of triethylammonium bicarbonate, the reservoir containing 4 l. of 1 *M* triethylammonium bicarbonate in 20% aqueous ethanol, the mixing chamber containing 4 l. of 0.001 *M* triethylammonium bicarbonate. About 18-ml. fractions were collected every 10 min. A small amount of uridine-3′ phosphate was first eluted (around fraction 150) followed by another minor peak (peak fraction 184) which has not been identified. The main peak corresponding to 5′-*O*-di-*p*-methoxytrityluridine-3′ phosphate was eluted in fractions 200–295. (A peak which appeared subsequently was assumed to be that of 2′,5′-bis-*O*-di-*p*-methoxytrityluridine-3′ phosphate.) Fractions 200–295 were pooled and evaporated at 10° using 1-octanol to prevent frothing. The evaporation was repeated several times by addition of pyridine in order to remove triethylammonium bicarbonate. The product thus obtained was homogeneous by paper chromatography. The yield as estimated spectrophotometrically (at *p*H 8) was 0.82 mmole (60%).

2′-*O*-Acetyluridine-3′ Phosphate *via* 5′-*O*-Tetrahydropyranyluridine-3′ Phosphate.—5′-*O*-Tetrahydropyranyluridine-3′ phosphate (ammonium salt, 0.28 mmole) was dissolved in cold water (5 ml.) and to the solution was added a previously prepared mixture of acetic anhydride (9 ml.) and pyridine (9 ml.). The total mixture was shaken rapidly for 30 min. with intermittent immersion in an ice–water-bath. The total solution was then concentrated under a high vacuum to remove most of pyridinium acetate. The residue was dissolved in water (20 ml.) and Dowex-50 (H^+) was gradually added until the *p*H fell to 2.5. The acidic solution was kept at room temp. for 7.5 hr.[38] Paper chromatography in solvent B at this stage showed the desired product 2′-*O*-acetyluridine-3′ phosphate to be 58%. Other products detected were a small amount of uridine-2′(3′) phosphate and some 5′-*O*-tetrahydropyranyluridine-2′(3′) phosphate (or uridine-2′,3′ cyclic phosphate). The resin was removed by filtration and the solution was evaporated at about 10° under a high vacuum to a small volume (about 2 ml.). Ten milliliters of an ice-cold mixture of ethyl alcohol–1 *M* ammonium acetate, *p*H 6.5 (5:2, v./v.) was added and the solution applied to the top of a cellulose column (100 cm. × 2.5 cm. dia.) equilibrated in the cold with the same solvent. Elution was carried out with the

(36) This was the period of time which, under the conditions of reactant concentrations used, brought about optimal monotritylation of the 5′-hydroxyl group in 2′-*O*-tetrahydropyranyluridine-3′ phosphate.

(37) The formation of this product will explain the fact that the yield (34%) of 5′-*O*-tetrahydropyranyluridine-3′ phosphate isolated above was lower than that present in the starting mixture of monotetrahydropyranyl derivatives.

(38) The half-life of the phosphate ring in 5′-*O*-tetrahydropyranyluridine-2′,3′ cyclic phosphate at *p*H 2.5 and room temp. was found to be about 85 min.

same solvent system at 4° at a flow rate of about 1 ml./min., 10-ml. fractions being collected. Pyridine appeared at around fraction 59 and a minor peak of nucleotidic material appeared at fraction 72. This peak contained a small amount of uridine-2',3' cyclic phosphate. The major peak corresponding to 2'-*O*-acetyluridine-3' phosphate appeared at around fraction 92. (Uridine-2'(3') phosphate appeared at fraction 132.) The appropriate fractions containing the desired product were combined and the total solution was passed through a column (25 cm. × 4 cm. dia.) of Dowex-50 (H^+) ion exchange resin in the cold. The total effluent and washings were concentrated at 5° under reduced pressure to remove acetic acid. The concentrate (about 75 ml.) was diluted with cold water (100 ml.) and the solution lyophilized. The resulting powder of 2'-*O*-acetyluridine-3' phosphate was homogeneous by paper chromatography (R_f's in table) and paper electrophoresis. Further characterization was performed as described below (test for absence of 2',3'-cyclic phosphate formation on treatment with DCC and preparation of methyl uridine-3' phosphate).

2'-*O*-Acetyluridine-3' Phosphate *via* 5'-*O*-Di-*p*-methoxytrityluridine-3' Phosphate.—Water (3 ml.) was added to a pyridine (12 ml.) solution of 5'-*O*-di-*p*-methoxytrityl-uridine-3' phosphate (0.78 mmole of triethylammonium salt). The solution was cooled in ice-water and acetic anhydride (10 ml.) was added with rapid stirring. The solution was then allowed to warm up to room temp. (1-5 hr.) and then evaporated at 10° to remove pyridinium acetate. The process of evaporation from aqueous pyridine was repeated several times. Water was then added and the *p*H of the solution brought rapidly to 2.0-2.4 with Dowex-50 ion exchange resin (H^+). After 4 min. at this *p*H (room temp.) the solution was rapidly filtered from the resin and the total filtrate frozen. Total time taken for acidic treatment and freezing was 6-7 min. The frozen solution was lyophilized and the resulting powder was dissolved in a small volume of an ice-cold mixture of ethyl alcohol-1 *M* ammonium acetate, *p*H 6.5 (5:2, v./v.). The solution was applied to the top of a cellulose powder column (100 cm. × 2.5 cm. dia.) previously equilibrated with the same solvent at 5°. Fractions of about 13-ml. volume were collected at a flow rate of 0.8 ml./min. The major peak corresponding to 2'-*O*-acetyluridine-3' phosphate was eluted in fractions 197-217 and the eluate was immediately passed through a column (3 cm. × 25 cm.) of Dowex-50 ion exchange resin (H^+) at 5° to remove the ammonium ions. The acidic effluent from this column was evaporated to a small volume at 10°, the concentrate was diluted with ice-cold water (50 ml.) and the solution lyophilized. The lyophilized powder was redissolved in a small volume of water and the solution repassed through a small column (10 cm. × 1 cm.) Dowex-50 (H^+) resin. The ice-cold effluent was lyophilized and the resulting powder was dissolved in anhydrous pyridine. The product was shown to be pure 2'-*O*-acetyluridine-3' phosphate[39] by paper chromatography and paper electrophoresis.

The Reaction of Mononucleotide Derivatives with DCC.—The course of reaction of 5'-*O*-acetyluridine-2' (or 3') phosphate, 5'-*O*-tetrahydropyranyluridine-3' phosphate and 2'-*O*-acetyluridine-3' phosphate with DCC in aqueous pyridine at room temp. was used to show the presence or absence of an adjacent hydroxyl group. The standard conditions used were: The nucleotide (2-3 μmoles) in a mixture of pyridine (1 ml.) and water (0.2 ml.) was treated with DCC (150 mg.) at room temp. Aliquots were removed at intervals and subjected to paper electrophoresis at *p*H 7.1 or examined by paper chromatography in solvent A. Wherever cyclization to form 2',3'-cyclic phosphate formation was possible, this reaction was complete in about 1 hr. at room temp. The time of reaction mostly limited to this, since longer reaction periods gave some side reactions.

Methyl Uridine-3' Phosphate: (a) From 2',5'-Di-*O*-acetyluridine-3' Phosphate.—Methyl alcohol (1 ml.) was added to a dry pyridine (0.3 ml.) solution of 2',5'-di-*O*-acetyluridine-3' phosphate (3.24 μmoles) and the total solution was then treated with DCC (0.150 g.). After 18 hr. at room temp., pyridine (1 ml.) and water (1 ml.) were added and the mixture extracted with light petroleum ether (3 × 1 ml.). The aqueous pyridine solution was kept at room temperature for 6 hours and then evaporated to a gum. To this was added ammonium hydroxide (2 ml. of 7 *N*) and some glass beads. The mixture was briefly shaken to break up clumps of dicyclohexylurea and then allowed to stand at room temperature for 6 hr. The dicyclohexylurea was removed by filtration and the filtrate was chromatographed in solvent B. Methyl uridine-3' phosphate[40] (R_f 0.69), the sole product, was eluted with water and the eluate passed through a small column of Dowex-50 ion exchange resin (H^+) in the cold. The effluent and washings were quickly frozen and lyophilized. The lyophilized powder was incubated with pancreatic ribonuclease under the standard conditions. When the incubation mixture was examined by paper electrophoresis at *p*H 7.1, the only ultraviolet-absorbing product noted was uridine-3' phosphate.

(b) **From 2'-*O*-acetyluridine-3' Phosphate.**—A mixture of 2'-*O*-acetyluridine-3' phosphate (3 μmoles), pyridine (0.5 ml.), methyl alcohol (1.0 ml.) and DCC (0.100 g.) was kept at room temperature for 9 hr. The work-up was exactly as described above. Methyl uridine-3' phosphate was obtained quantitatively, and when incubated with pancreatic ribonuclease under the standard conditions complete degradation to uridine-3' phosphate occurred.

Uridylyl-(3'→5')-uridine.—To an anhydrous pyridine solution (0.3 ml.) of 2'-*O*-acetyluridine-3' phosphate (0.037 mmole, pyridinium salt) and 2',3'-di-*O*-benzoyluridine (0.033 g.) was added DCC (0.150 g.) and the sealed reaction mixture was kept at room temp. for 4 days. Water (0.5 ml.) was then added and the solution extracted with petroleum ether (3 × 1 ml.). The aqueous pyridine solution was kept at room temp. for 18 hr. and then was treated with 9 *N* ammonium hydroxide (3 ml.) for 6 hr. The precipitate of dicyclohexylurea was removed by filtration and the total solution chromatographed in solvent A. Three nucleotidic bands were observed, in addition to that of uridine. The major band (65%) was that of uridine monophosphate, but it was contaminated by a number of polymeric products present in small amounts. (These corresponded presumably to di- and trinucleotides and related compounds to be expected by self-condensation of the starting material.) Uridylyl-(3'→5')-uridine, the second band from the original mixture, was obtained in 5.5% yield. On incubation with pancreatic ribonuclease it was completely degraded to uridine-3' phosphate and uridine. The third band obtained in the above condensation was that of uridine-3',5' cyclic phosphate (29%). It was characterized by extensive paper chromatography and by paper electrophoresis. Incubation with pancreatic ribonuclease gave mostly the starting material, there being formed a trace (<2%) of uridine-3' phosphate. The latter presumably arose from uridine-2',3' cyclic phosphate[41] which might have been a contaminant in the 3',5'-cyclic phosphate.

Uridylyl-(3'→5')-adenosine.—An anhydrous pyridine solution (0.5 ml.) of 2',5'-di-*O*-acetyluridine-3' phosphate (50 μmoles of pyridine salt) and N,N-2'-*O*,3'-*O*-tetrabenzoyladenosine (0.068 g., 0.1 mmole) was treated with DCC (0.150 g.) and the mixture kept sealed at room temp. for 4 days. Water (0.5 ml.) was added and the mixture extracted with petroleum ether (3 × 1 ml.). The aqueous pyridine solution was kept at room temp. overnight and then treated with 1 ml. of concd. ammonium hydroxide at room temp. for 8 hr. Ammonia was then removed by evaporation, the residual gum was dissolved in water and the insoluble dicyclohexylurea was removed by filtration. The total solution was then chromatographed on Whatman 44 paper using solvent A. The yield of uridylyl-(3'→5')-adenosine was 84% as based on the nucleotide used. It was characterized by the methods described in the previous papers.[2]

Polymerization of 2'-*O*-Acetyluridine-3' Phosphate Using DCC.—2'-*O*-Acetyluridine-3' phosphate (0.0625 mmole, pyridine salt[42]) was carefully evaporated in the cold with anhydrous pyridine (3 × 0.5 ml.) to remove last traces of water. The anhydrous residue was then dissolved in dry pyridine (0.1 ml.) and DCC (0.1 g.) was added along with several glass beads. Upon the addition of DCC an oil separated from solution and the total mixture was shaken in the presence of glass beads for 2 days at room temperature. A further amount of DCC (0.05 g.) was then added and the mixture shaken for an additional period of 3 days. After this time, a solution of aqueous pyridine (2 ml. of pyridine + 1 ml. of water) was added and the solution was extracted immediately with petroleum ether (3 × 1 ml.) to remove DCC. The precipitated dicyclohexylurea was removed by filtration and the filtrate after standing at room temperature for 8 hours was evaporated to a gum. This was dissolved in 3 ml. of ammonium hydroxide (10 *M*) and kept at room temperature for 7 hours. After this time, the solution was filtered and the filtrate evaporated to dryness. The residue was dissolved in water (10 ml.) and the *p*H was brought to 9.5 with ammonium hydroxide. A part (corresponding to 55.0 μmoles of starting material) of this material was chromatographed on a DEAE-cellulose (bicarbonate form) column (50 cm. × 1 cm. dia.). The column was eluted with a linear gradient of triethylammonium bicarbonate (*p*H 7.5.). The mixing vessel and the salt vessel contained, respectively, 2 l. of water and 2 l. of 0.3 *M* triethylammonium bicarbonate. A flow rate of 1.5 ml./min. was maintained and 15-17-ml. fractions were collected. After fraction 96, by which time

(39) The exact yield at this stage is not known but it is estimated to be in the range of 40%.

(40) It is of interest to note that this diester was found stable to treatment with concentrated ammonium hydroxide for at least 3.5 hr.

(41) In the preparations of 2',5'-di-*O*-acetyluridine-3' phosphate which have been stored in dry pyridine for periods of weeks, a very small amount of hydrolysis to 5'-*O*-acetyluridine-3' phosphate could be expected and the latter on activation with DCC would form the 2',3'-cyclic phosphate. The latter does not appear to cause any complication, especially if its amount is insignificant. in the nature of the products. This is because the 2',3'-cyclic phosphate and pyrophosphate derived from it would not be expected to be adequately powerful phosphorylating agents.

(42) It is important to make sure that any strong base such as triethylamine is absent. The presence of the latter inhibits polymerization.[28]

six peaks had been eluted, the total nucleotidic material was eluted with 1 *M* triethylammonium bicarbonate. The 1 *M* eluate (about 30% of material applied to the column) was evaporated to dryness and the process of dissolution in water and evaporation repeated to remove most of the salt. The total mixture was then chromatographed on a 9″ wide strip Whatman No. 40 paper using solvent C. Each of the peaks obtained up to fraction 96 was also recovered by evaporation of the appropriate fractions. Characterization of the products recovered from the different peaks and the chromatogram was as follows.

C_5′,-Pyridinium uridine-3′ phosphate (XX) (4.4% was in fractions 28–31, peak 2), was eluted at a 0.05 *M* triethylammonium bicarbonate concentration; ultraviolet absorption: λ_{max} 260 mμ, shoulder at 267 mμ; λ_{min} 232 mμ in water; λ_{max} 260 mμ shoulder at 267 mμ; and λ_{min} 243 mμ at *p*H 11.5. Paper chromatographic and electrophoretic mobilities are in Tables I and II.

Uridine-3′,5′ cyclic phosphate (7.5%) constituted peak 3, being eluted in fractions 32–34. The paper chromatographic and electrophoretic mobilities are in Tables I and II. In its behavior to acid and alkali and in its resistance to pancreatic ribonuclease, the product was identical with the sample synthesized earlier.[25]

Uridine-3′ phosphate (9.7%) was present in peak 4 (fractions 42–52) being eluted at 0.079 *M* triethylammonium bicarbonate concentration.

Uridine cyclic dinucleotide (5.2%) was present in peak 5 (fractions 58–64) being eluted at 0.082 *M* triethylammonium bicarbonate concentration. The paper chromatographic and electrophoretic mobilities (Tables I and II) were consistent with its structure. Treatment with bacterial phosphomonoesterase under the standard conditions did not alter its electrophoretic mobility at *p*H 7.1. Incubation with pancreatic ribonuclease caused only partial degradation to uridine-3′ phosphate under the conditions which caused complete degradation of the dinucleotide uridylyl-(3′→5′)-uridine-3′ phosphate to the mononucleotide. Treatment with 0.5 *N* sodium hydroxide at 25° caused only about 20% hydrolysis in 6 hr. and about 65–70% in 54 hr., while uridylyl-(3′→5′)-uridine was completely hydrolyzed in 6 hr. under the above conditions. The main product from the cyclic dinucleotide on alkaline hydrolysis was uridine-2′(3′) phosphate, there being detected a small amount of an intermediate, presumably linear uridine dinucleotide.

Uridylyl-(3′→5′)-uridine-3′ phosphate (XVIII, *n* = 0) was present in peak 6, being eluted in fractions 84–96 (0.091 *M* triethylammonium bicarbonate concentration). The position of elution, paper chromatographic and paper electrophoretic properties (Tables I and II) were all consistent with its structure. Treatment with bacterial phosphomonoesterase caused complete conversion to uridylyl-(3′→5′)-uridine. The latter was identical in paper electrophoretic mobility with a synthetic sample and gave, after incubation with pancreatic ribonuclease, uridine-3′ phosphate and uridine in equal amounts. The dinucleotide itself was completely degraded to uridine-3′ phosphate on incubation with pancreatic ribonuclease.

Uridine Cyclic Trinucleotide (XIX, *n* = 1).—The material eluted by 1 *M* triethylammonium bicarbonate after elution of the preceding compound, uridylyl-(3′→5′)-uridine-3′ phosphate, was separated by paper chromatography in solvent C on a sheet of paper (Whatman No. 44). The cyclic trinucleotide constituted a major band[43] (R_f 0.25, solvent C) of the 1 *M* fraction (about 3.5% of total polymeric mixture). Its paper chromatographic and electrophoretic properties are given in Tables I and II. The electrophoretic mobility was almost identical with the previously synthesized thymidine cyclic dinucleotide. It was resistant to the action of the alkaline phosphomonoesterase. Degradation with pancreatic ribonuclease gave a product which was identified as uridine-3′ phosphate. (Sufficient material was not available for a comparative rate study with known substrates.)

Uridylyl-(3′→5′)-uridylyl-(3′→5′)-uridine-3′ phosphate (XVIII, *n* = 1) was present (about 2% of total nucleotidic material) in a band traveling slower (R_f 0.15, solvent C) than the cyclic trinucleotide. Its chromatographic properties are in Tables I and II. On incubation with the alkaline phosphomonoesterase it was completely converted to a product (R_f 0.25 in solvent C) which was identified as uridylyl-(3′→5′)-uridylyl-(3′→5′)-uridine. Degradation of the latter with pancreatic ribonuclease gave uridine-3′ phosphate and uridine in a ratio close to 2. The trinucleotide itself on incubation with pancreatic ribonuclease gave only uridine-3′ phosphate.

Higher Oligonucleotides.—Some 3% of the total polymeric mixture did not leave the origin on paper chromatography in solvent C as used above for separation of the di- and trinucleotides. This material evidently consisted of several components as shown by paper electrophoresis and included the oligonucleotides higher than those characterized above.

(43) This product was further purified by paper electrophoresis at *p*H 7.1. In this way two minor impurities were removed.

[CONTRIBUTION FROM THE INSTITUTE FOR ENZYME RESEARCH, UNIVERSITY OF WISCONSIN, MADISON, WIS., AND THE CHEMISTRY DIVISION OF BRITISH COLUMBIA RESEARCH COUNCIL, VANCOUVER, CAN.]

Studies on Polynucleotides. XX.[1] Amino Acid Acceptor Ribonucleic Acids (1). The Synthesis and Properties of 2′(or 3′)-O-(DL-Phenylalanyl)-adenosine, 2′(or 3′)-O-(DL-Phenylalanyl)-uridine and Related Compounds[2]

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The reaction of carbobenzyloxy-DL-phenylalanine with dicyclohexylcarbodiimide gave an excellent yield of the corresponding symmetrical anhydride III, which was isolated in a crystalline form and characterized. The pyridine-catalyzed reaction of III with 5′-O-tri-*p*-methoxytrityluridine followed by an acidic treatment gave mono-O-(carbobenzyloxy-DL-phenylalanyl)-uridine, which was, presumably, a mixture of the 2′- and 3′-isomers. Palladium-catalyzed hydrogenolysis of the latter afforded 2′(or 3′)-O-(DL-phenylalanyl)-uridine. Analogous reaction of III with 5′-O-trityladenosine gave both 3′-O-(carbobenzyloxy-DL-phenylalanyl)-5′-O-trityladenosine and the corresponding 2′-isomer which were separated by chromatography on a silicic acid column. The isomers were found to undergo interconversion under mildly acidic or basic catalysis. It proved possible, however, to determine the orientation of the protected aminoacyl residue in the products by phosphorylation and identification of the resulting adenosine-2′ or -3′ phosphate. Removal of the protecting groups from the above derivatives gave DL-phenylalanyladenosine which was, presumably, a mixture of the 2′- and 3′-isomers. The rate of hydrolysis of the aminoacyl nucleoside was determined in *p*H 7 phosphate buffer. The half-life at 25° was 48 min., while that at 34° was 22 min. The possible causes for the great lability of the aminoacyl linkage in the adenosine ester are discussed. Furthermore, the present work suggests the rapid migration of the amino acyl groups between the 2′- and 3′-hydroxyl groups of terminal adenosine in amino acid acceptor ribonucleic acids.

The first steps in the enzymatic synthesis of a polypeptide chain are the activation of α-amino acids, by reaction with adenosine-5′ triphosphate, to form the mixed anhydrides of the type I and the subsequent transfer of the aminoacyl groups to the terminal adenosine residues (partial structure II)[4] of relatively small molecular weight ribonucleic acids, designated variously as soluble, transfer, or amino acid acceptor

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(4) For the system of short hand representation of polynucleotides see H. G. Khorana "Some Recent Developments in the Chemistry of Phosphate Esters of Biological Interest," John Wiley and Sons, Inc., New York, N. Y., 1961. Chapter 5.