[CONTRIBUTION FROM THE INSTITUTE FOR ENZYME RESEARCH, THE UNIVERSITY OF WISCONSIN, MADISON 6, WIS.]

Studies on Polynucleotides. XXVIII.[1] The Specific Synthesis of $C_{3'}$–$C_{5'}$-Linked Ribooligonucleotides (4).[2] The Stepwise Synthesis of Uridylyl-(3′ → 5′)-adenylyl-(3′ → 5′)-uridylyl-(3′ → 5′)-uridine[3]

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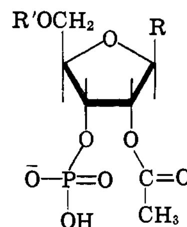
The reaction of pyridinium uridine-3′ phosphate with *p*-anisyldiphenylmethyl (monomethoxytrityl) chloride in pyridine followed by chromatography gave 5′-O-monomethoxytrityluridine-3′ phosphate (70%). Acetylation with acetic anhydride in the presence of an excess of tetraethylammonium acetate gave quantitatively the 2′-O-acetyl derivative. Condensation of this protected nucleotide with an excess of N,2′-O,3′-O-tribenzoyluridine in the presence of dicyclohexylcarbodiimide (DCC) followed by a mild acidic treatment gave 2′-O-acetyluridylyl-(3′ → 5′)-N,O$^{2'}$,O$^{3'}$-tribenzoyluridine (IV) which was isolated pure either by partition or by anion-exchange chromatography. Condensation of the latter with an excess of N,2′-O,3′-O-triacetyladenosine-3′ phosphate and with 2′,5′-di-O-acetyluridine-3′ phosphate in the presence of DCC followed by an ammoniacal treatment gave, respectively, adenylyl-(3′ → 5′)-uridylyl-(3′ → 5′)-uridine and uridylyl-(3′ → 5′)-uridylyl-(3′ → 5′)-uridine in good yield. Condensation of IV with 5′-O-monomethoxytrityl-2′-O-acetyl-N-benzoyladenosine-3′ phosphate followed by an acidic treatment and anion-exchange chromatography gave 2′-O-acetyl-N-benzoyladenylyl-(3′ → 5′)-2′-O-acetyluridylyl-(3′ → 5′)-N,2′-O,3′-O-tribenzoyluridine (VIII). Condensation of the latter with an excess of 2′,5′-di-O-acetyluridine-3′ phosphate in the presence of DCC followed by an ammoniacal treatment gave the tetranucleotide uridylyl-(3′ → 5′)-adenylyl-(3′ → 5′)-uridylyl-(3′ → 5′)- uridine. The synthetic unprotected tri- and tetranucleotides were shown enzymically to consist exclusively of $C_{3'}$–$C_{5'}$ internucleotidic linkages.

Concurrently with the program of synthetic work in the deoxyribopolynucleotide field,[4–6] the problems of the specific synthesis of $C_{3'}$–$C_{5'}$ linked ribopolynucleotides have been under intensive study in this Laboratory.[2a,2b,4] In an earlier paper, it was concluded that the only practically feasible approach to the synthesis of the $C_{3'}$–$C_{5'}$ inter-ribonucleotidic linkage is that in which a suitably protected rubonucleoside-3′ phosphate is condensed with another protected nucleoside or nucleotide which contains a free 5′-hydroxyl group. The key intermediates developed in early work were those in which the 2′-hydroxyl group of the ribonucleoside-3′ phosphates was protected by the acid-labile tetrahydropyranyl group.[2a,2b,7] Subsequent rigorous examination showed that this protecting group was not completely satisfactory and it was abandoned in favor of the alkali-labile acetyl group.[2c,8] In further work, a method for the direct preparation of the 2′-O-acetylribonucleoside-3′ phosphates in quantitative yield from the parent nucleotides was developed.[2c,9] This basic approach in which an alkali-labile group is used to protect the 2′-hydroxyl group is now being incorporated into schemes for the stepwise synthesis of specific ribopolynucleotides. The present communication records results of the initial phase of this work which has led to the synthesis of the trinucleotides, adenylyl-(3′ → 5′)-uridylyl-(3′ → 5′)-uridine and uridylyl-(3′ → 5′)-uridylyl-(3′ → 5′)-uridine, and of the tetranucleotide, uridylyl-(3′ → 5′)-adenylyl-(3′ → 5′)-uridylyl-(3′ → 5′)-uridine. A brief report of a part of this work has already appeared.[10] An accompanying paper deals further with the problem of ribopolynucleotide synthesis by the polymerization of suitably protected ribonucleotides.[11]

The use of an alkali-labile protecting group for the 2′-hydroxyl group of ribonucleoside-3′ phosphates dictated the use of a group such as a trityl group to protect the 5′-hydroxyl group (typical structure of the derivative I) and the stepwise synthesis of ribooligonucleotides was envisaged as proceeding by selective exposure of the 5′-hydroxyl group of the growing oligonucleotide chain by a mild acidic step followed by a repeat of the condensation step with a protected



I, R = protected purine or pyrimidine
R′ = trityl or substituted trityl

nucleotide of the type I. The direct reaction of a ribonucleoside-3′ phosphate with di-*p*-methoxytritylchloride[2a] was therefore studied, the nucleotide chosen being adenosine-3′ phosphate. A mono-di-*p*-methoxytrityl derivative was indeed obtained as a major product (see accompanying paper[11]). That the latter was 5′-O-di-*p*-methoxytrityladenosine-3′ phosphate was shown by its spectral properties and by its hydrolysis on acidic treatment to adenosine-3′ phosphate only. The latter result showed that no migration of the phosphoryl group to the 2′-position was involved. The same reaction was then applied to uridine-3′ phosphate, as has been previously described for the preparation of 2′-O-acetyluridine-3′ phosphate.[2c] For the present purpose of stepwise synthesis, the relatively more stable 5′-O-monomethoxytrityluridine-3′ phosphate was prepared by reaction of the parent nucleotide with monomethoxytrityl chloride under carefully controlled conditions. The desired product was purified by

(1) Paper XXVII: H. Schaller and H. G. Khorana, *J. Am. Chem. Soc.*, **85**, 3841 (1963).

(2) The previous three papers in this series which deal directly with this topic are: (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), N.Y.

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

(4) 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.

(5) For stepwise synthesis of deoxyribopolynucleotides see H. Schaller and H. G. Khorana, *J. Am. Chem. Soc.*, **85**, 3828 (1963), and earlier references cited therein.

(6) For the polymerization of mononucleotides see H. G. Khorana, J. P. Vizsolyi, and R. K. Ralph, *ibid.*, **84**, 414 (1962), and earlier references cited therein.

(7) Two other groups of workers have more recently used the tetrahydropyranyl group as a blocking group for the 2′-hydroxyl group in ribonucleoside-3′ phosphates. See, *e.g.*, J. Smrt and F. Sorm, *Collection Czechoslov. Chem. Commun.*, **28**, 61 (1963), and F. Cramer and K. H. Scheit, *Angew. Chem. Intern. Ed. Engl.*, **1**, 510 (1962).

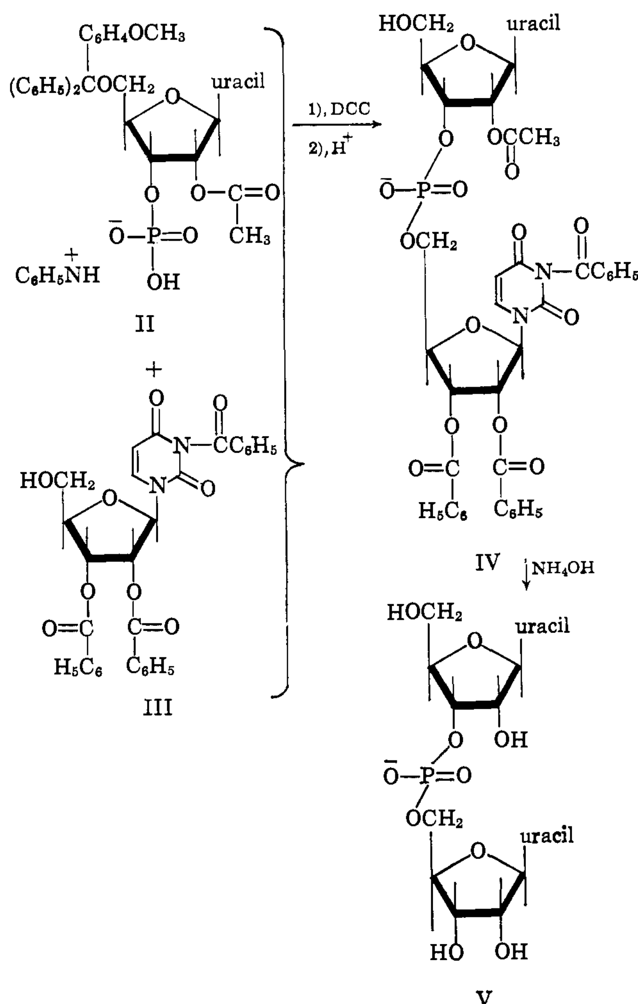
(8) D. H. Rammler and H. G. Khorana, *Biochem. Biophys. Research Commun.*, **7**, 147 (1962); **8**, 61 (1962).

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

(10) Y. Lapidot and H. G. Khorana, *J. Am. Chem. Soc.*, **85**, 1363 (1963).

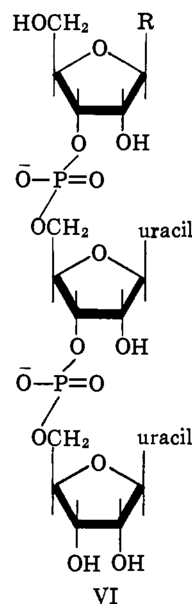
(11) Y. Lapidot and H. G. Khorana, *ibid.*, **85**, 3857 (1963).

anion exchange chromatography, the yield being 70%. Acetylation with acetic anhydride in the presence of an excess of tetraethylammonium acetate[2c] gave quantitatively the 2′-O-acetyl derivative II which was isolated as a white powder (pyridine salt) by precipitation from an excess of ether.[12]



The protected nucleoside used in the present work was N,2′-O,3′-O-tribenzoyluridine (III).[13] Condensation of II with an excess of III in the presence of DCC followed by an acidic treatment gave the desired protected derivative IV, which was isolated pure in good yield by partition chromatography on a cellulose powder column using an acidic solvent system. A more practical method for the isolation of IV was later found in anion exchange chromatography at 2° using DEAE–cellulose in the carbonate form. Compound IV was characterized as pure by paper chromatography, it being made certain that the 2′-O-acetyl group, the most labile group in the product, was not affected during the isolation procedure. (The loss of the acetyl group in IV resulted in the appearance of a spot on paper chromatograms with R_f slightly less than that of IV.) As might be expected, IV was fully resistant to the action of the pancreatic ribonuclease. On the other hand, uridylyl-(3′ → 5′)-uridine (V) obtained from IV after an ammoniacal treatment was completely degraded by the enzyme to uridine-3′ phosphate and uridine.

In initial experiments on the further condensation of IV with protected ribonucleoside-3′ phosphates, 2′,5′-di-O-acetyluridine-3′ and N,2′-O,3′-O-triacetyladenosine-3′ phosphates were used. The reaction of IV with 4 molar equivalents of either of these nucleotides in the presence of DCC followed by an ammoniacal treatment gave, respectively, uridylyl-(3′ → 5′)-uridylyl-(3′ → 5′)-uridine (VI, R = uracil) and adenylyl-(3′ → 5′)-uridylyl-(3′ → 5′)-uridine (VI, R = adenine). The yield in both syntheses was around 75% as based on the amount of IV used. Both of the products (VI, R = uracil or adenine) were checked carefully for the exclusiveness of the $C_{3'}$–$C_{5'}$ internucleotidic linkages in them. Thus the trinucleotide VI (R = adenine) was completely degraded to adenosine-3′ phosphate, uridine-3′ phosphate, and uridine by the *Lactobacillus acidophilus* phosphodiesterase,[14] while the uridine oligonucleotide VI (R = uracil) was completely degraded[15] by the pancreatic ribonuclease to uridine-3′ phosphate and uridine.



In an accompanying paper is described the preparation of pyridinium 5′-O-monomethoxytrityl-2′-O-acetyl-N-benzoyladenosine-3′ phosphate (VII), an intermediate in the preparation of N-benzoyl-2′-O-acetyladenosine-3′ phosphate. With the aim of the synthesis of a tetranucleotide as depicted in Chart I, VII was condensed with the protected derivative IV. A mild acidic treatment of the initially formed fully protected trinucleotide gave VIII which was purified by anion exchange chromatography on a DEAE–cellulose (carbonate) column. The yield of VIII as based on IV was about 72%, a fourfold excess of VII having been used. The successful isolation of VIII in a chromatographically homogeneous form and the previous isolation of IV encourage the hope that the same type of methodology could be applied to the isolation of larger sized polynucleotide intermediates.

The condensation of VIII with a sevenfold excess of 2′,5′-di-O-acetyluridine-3′ phosphate under the standard conditions followed by an ammoniacal treatment

(12) It should be noted that at the end of the acetylation reaction, II is initially present mainly as its mixed anhydride monoacetyl phosphate. The complete hydrolysis of this intermediate to II required conditions different from those found necessary previously in the case of 2′,5′-di-O-acetyluridine-3′ phosphate. This aspect is discussed elsewhere: C. Coutsogeorgopoulous and H. G. Khorana, paper in preparation.

(13) The full characterization and preparation of this derivative of uridine will be reported elsewhere: R. Lohrmann and H. G. Khorana, unpublished work; D. H. Rammler, private communication.

(14) W. Fiers and H. G. Khorana, *J. Biol. Chem.*, **238**, 2781 (1963).

(15) Enzymic degradation of VI (R = adenine) was performed using 9.6 optical density units (260 mμ) while that of VI (R = uracil) was performed using an amount containing 0.7 μmole of mononucleotide. By application of the total product on paper chromatograms, the degradation was found to be complete. At the levels tested, the synthetic products are concluded to have exclusively the $C_{3'}$–$C_{5'}$, internucleotidic linkages.

CHART I

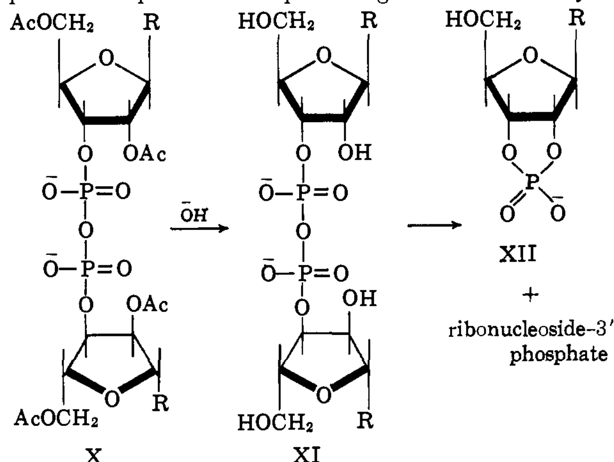
STEPWISE SYNTHESIS OF URIDYLYL-(3' → 5')-ADENYLYL-(3' → 5')-URIDYLYL-(3' → 5')-URIDINE

VII + IV —1, DCC; 2, H^+→ VIII —1, DCC; 2, NH_4OH; 3, chromatography→ IX

VIII —NH_4OH→ VI

gave the tetranucleotide, uridylyl-(3' → 5')-adenylyl-(3' → 5')-uridylyl-(3' → 5')-uridine (IX) which was isolated by chromatography on an ion exchange column; IX was characterized fully for homogeneity and for its susceptibility to enzymic degradation. The latter went to completion showing again the exclusiveness of the $C_{3'}$–$C_{5'}$ linkages.

During chromatography of the protected oligonucleotides, the pyrophosphates corresponding to the protected mononucleotides, for example X, were encountered. Compounds of this type would be expected to be labile in the absence of the acetyl groups on the 2'-hydroxyl groups, the degradation proceeding *via* the facile cyclic phosphate formation in such systems.[16] This was further demonstrated to be so by treatment of X with aqueous ammonia at room temperature. Paper chromatography showed the products to consist of uridine-2',3' cyclic phosphate and uridine-3' phosphate. It seems certain that the rate-determining step in the formation of the cyclic phosphate and the free nucleotide is in fact the removal of the 2'-O-acetyl group. Furthermore, in an unpublished experiment by R. J. Young in this Laboratory, pyridinium uridine-3' phosphate was treated in dry pyridine with methyl phosphoromorphilidate, in an attempt to prepare P^1-methyl-P^2-uridine-3' pyrophosphate. Direct paper electrophoresis at pH 5 again showed only a



trace of a product corresponding to the pyrophosphate. The major ultraviolet-absorbing product was uridine-2',3' cyclic phosphate.

(16) H. G. Khorana, ref. 4, Chapter 3. Also see H. G. Khorana, G. M. Tener, R. S. Wright, and J. G. Moffatt, *J. Am. Chem. Soc.*, **79**, 430 (1957).

From the above, it is clear that in the present approach to oligonucleotide synthesis, when the work-up involves an ammoniacal treatment to remove the acetyl groups, ribonucleoside-2′,3′ cyclic phosphates would be expected to be formed from the surviving pyrophosphates of the type X by the manner illustrated in the formulas X–XII.

Concluding Remarks.—The present work has reported on the development of principles for the stepwise synthesis of ribopolynucleotides. The synthesis accomplished was that of a tetranucleotide and contained uridine residues and only one residue of a different nucleoside, namely adenosine. The work represents only an initial phase of a long range attack on the problems of the synthesis of ribopolynucleotides containing different nucleosides in predetermined sequences. The problems of the specific protecting groups for other nucleosides will be discussed in a forthcoming paper.[17]

The key intermediates in the present work contain the alkali-labile acetyl group on the 2′-hydroxyl groups and an acid-sensitive group is used to protect the 5′-hydroxyl group. A successive mild acidic treatment thus selectively exposes the 5′-hydroxyl group and the chain elongation occurs by a repeat condensation with a suitably protected ribonucleoside-3′ phosphate. Consistently high yields with respect to the oligonucleotide component are sustained in the condensation reactions by providing a proportionately large excess of the mononucleotide component. These are also the principles which have been developed for the stepwise synthesis of deoxyribopolynucleotides[18] with the important difference that the total strategy there involves the growth of a chain so that the repeat condensation occurs between the terminal 3′-hydroxyl group of the oligonucleotide component and the 5′-phosphoryl group of a suitably protected deoxyribonucleoside-5′ phosphate. The direction of the growth of the polynucleotide chains by chemical synthesis is thus opposite for the ribo- and the deoxyribopolynucleotides.

Experimental

General Methods.—Paper chromatography was performed by the descending technique using Whatman No. 1 or 40 paper. The solvent systems used were: solvent A, isopropyl alcohol–concentrated ammonia–water, 7:1:2 (v./v.); solvent B, ethyl alcohol–1 M ammonium acetate (pH 7.5) 7:3 (v./v.); solvent C, n-butyl alcohol–acetic acid–water, 5:2:3 (v./v.); solvent D, n-propyl alcohol–ammonia–water, 55:10:35. The R_f's of different compounds are given in Table I.

Paper electrophoresis was performed in a high voltage (4000 v.) apparatus in which the paper was immersed in a water-cooled high boiling petroleum fraction (Varsol). The relative electrophoretic mobilities of different compounds are given in Table II.

The trityl-containing compounds were made visible on paper chromatograms by spraying the chromatograms with dilute perchloric acid as described earlier and warming in an oven.[19] The compounds containing mono-p-methoxytrityl group appeared yellowish orange, those containing di-p-methoxytrityl group appeared orange-red.

Degradation by the pancreatic ribonuclease was carried out as described earlier; the amounts of the nucleotidic materials used in individual experiments are indicated in appropriate places. Degradation by the *Lactobacillus acidophilus* R-26 phosphodiesterase was performed using a preparation made by Dr. Walter Fiers and described elsewhere.[14] The incubation mixture contained 0.1 ml. of solution of the oligonucleotide (10 optical density units at 260 mμ), 0.01 ml. of 1 M TRIS chloride buffer (pH 8), and 0.05 ml. of the enzyme preparation which had been previously standardized against known substrates. After incubation for 4 hr. at 37°, the total mixture was chromatographed on paper. Chromatography was mostly performed in solvent B which separated urine-3′ phosphate from adenosine-3′ phosphate and uridine from the nucleotides.

(17) R. Lohrmann and H. G. Khorana, forthcoming paper.

(18) T. M. Jacob and H. G. Khorana, unpublished work.

(19) H. Schaller, G. Weimann, B. Lerch, and H. G. Khorana, *J. Am. Chem. Soc.*, **85**, 3821 (1963).

TABLE I

PAPER CHROMATOGRAPHY OF DIFFERENT COMPOUNDS (SOLVENTS IN TEXT)

Compound[a]	R_f Solv. A	Solv. B	Solv. C	Solv. D
Uridine-3′ phosphate	0.12	0.28	0.16	0.32
2′-O-Acetyluridine-3′ phosphate		.46	.26	
2′,5′-Di-O-acetyluridine-3′ phosphate		.54	.36	
N,2′,5′-Triacetyladenosine-3′ phosphate		.59	.38	
2′,5′-Di-O-acetyladenosine-3′ phosphate		.49	.36	
5′-O-Monomethoxytrityluridine-3′ phosphate	0.52	.82		
2′,5′-Bis-O-monomethoxytrityluridine-3′ phosphate	0.74			
2′-O-Acetyl-5′-O-monomethoxytrityluridine-3′ phosphate		0.88		
2′-O-Acetyluridylyl-(3′ → 5′)-N,2′,3′-tribenzoyluridine			0.70[b] .60[c]	
Uridylyl-(3′ → 5′)-N,2′,3′-tribenzoyluridine			.51[c]	
Uridylyl-(3′ → 5′)-uridine	0.24		.09	0.34
Uridylyl-(3′ → 5′)-uridylyl-(3′ → 5′)-uridine	.065		.03	.23
Adenylyl-3′ → 5′)-uridylyl-(3′ → 5′)-uridine	.065		.027	.23
N-Benzoyl-2′-O-acetyladenylyl-(3′ → 5′)-2′-O-acetyluridylyl-(3′ → 5′)-N,2′,3′-tribenzoyluridine			.82	
Uridylyl-(3′ → 5′)-adenylyl-(3′ → 5′)-uridylyl-(3′ → 5′)-uridine				.13
P^1,P^2-(N-Benzoyl-2′-O-acetyladenosine-3′) pyrophosphate		0.80		
P^1,P^2-(2′-O-Acetyluridine-3′) pyrophosphate		.78		

[a] Most of the compounds were used as the pyridine salts. The R_f's were not affected when ammoniacal solvents were used. In the acidic solvent C, R_f's of pyridine and ammonium salts are likely to differ from each other. [b] Pyridine salt. [c] Ammonium salt.

TABLE II

PAPER ELECTROPHORETIC MOBILITIES OF DIFFERENT COMPOUNDS

Compound	Mobility relative to uridine-3′ phosphate pH 7.1[a]	pH 2.1[b]
Uridine-3′ phosphate	1.0	1.0
Adenosine-3′ phosphate	0.85	0.0
Uridylyl-(3′ → 5′)-uridine	.31	0.83
Uridylyl-(3′ → 5′)-uridylyl-(3′ → 5′)-uridine	.61	1.0
Adenylyl-(3′ → 5′)-uridylyl-(3′ → 5′)-uridine	.53	0.52
Uridylyl-(3′ → 5′)-adenylyl-(3′ → 5′)-uridylyl-(3′ → 5′)-uridine	.72	0.78

[a] The buffer used was phosphate (0.03 M), pH 7.1. [b] The buffer used was glycine hydrochloride (0.05 M), pH 2.1.

Pyridinium 2′,5′-Di-O-acetyluridine-3′ Phosphate.—Pyridinium uridine-3′ phosphate (1 mmole) and tetraethylammonium acetate (10 mmoles) were rendered anhydrous by repeated evaporation of pyridine. In the last evaporation, suction was continued until a viscous gum remained. Acetic anhydride (10 mmoles) was added and the sealed reaction mixture (clear solution) was kept at room temperature for 2 hr. Methyl alcohol (5 ml.) was added to consume the excess of acetic anhydride and after 15 min. at room temperature the solution was evaporated. Water (10 ml.) and pyridine (10 ml.) were added and the aqueous pyridine solution kept at room temperature for 1 hr. to hydrolyze the acetyl phosphate (mixed anhydride). The solution was then passed through a bed (50-ml. volume) of pyridinium Dowex-50 ion exchange resin. The total effluent and washings were co-evaporated with pyridine to a small volume and the solution re-evaporated after addition of pyridine. The concentrated pyridine solution (5–10 ml.) was added dropwise to anhydrous ether (200 ml.) under constant agitation. A fine white powder thus resulted. It was collected by centrifugation, washed with dry ether, and the powder dried and stored in a vacuum desiccator over phosphorus pentoxide. The yield of monopyridinium salt was 46 mg. (93%). The product was homogeneous by paper chromatography and paper electrophoresis (R_f's in Tables I and II).

N,2′-O,3′-O-Triacetyladenosine-3′ Phosphate was prepared exactly as described above for uridine-3′ phosphate except that the time of acetylation was increased to 3 days in order to complete the acetylation of the ring amino group. Completion of the latter reaction was followed by paper chromatography of an aliquot in the solvent B, N,2′,5′-triacetyladenosine-3′ phosphate being readily distinguishable from 2′,5′-di-O-acetyladenosine-3′ phosphate (Table I) and by its characteristic ultraviolet absorption spectrum (λ_{max} 272 mμ).

Pyridinium 5′-O-Monomethoxytrityluridine-3′ Phosphate.—To an anhydrous pyridine solution (30 ml.) of pyridinium uridine-3′ phosphate (2 mmoles) was added mono-*p*-methoxytrityl chloride (1.86 g., 6 mmoles) and the stoppered reaction mixture shaken at room temperature for 6 hr. Ammonium hydroxide (100 ml. of 0.1 *M*) was then added and the solution extracted with ether (4 × 50 ml.) to remove monomethoxytritanol. The aqueous solution was evaporated at low temperature and reduced pressure to a small volume (15 ml., addition of pyridine) and some ethyl alcohol was added to redissolve the cloudiness which appeared. The solution was placed on a DEAE–cellulose (carbonate) column (51 × 3.7 cm. diam.). The elution was carried out with a linear gradient of triethylammonium bicarbonate, the reservoir containing 4 l. of 0.5 *M* triethylammonium bicarbonate in 20% aqueous ethyl alcohol and the mixing vessel containing an equal volume of water. About 16-ml. fractions were collected at a flow rate of 1.6 ml./min. A small amount of uridine-3′ phosphate (4%) first appeared (around fraction 130) followed by another minor peak (peak in fraction 145) which remains unidentified. 5′-O-Monomethoxytrityluridine-3′ phosphate appeared in fractions 170–290. (A subsequent peak, fractions 300–340, contained 2′,5′-bis-O-monomethoxytrityluridine-3′ phosphate.) Fractions containing the desired product were pooled, evaporated at 10° under reduced pressure, pyridine being frequently added in the latter stages of evaporation. The evaporation from pyridine was repeated several times to ensure complete removal of triethylammonium bicarbonate. The product thus obtained was homogeneous by paper chromatography. The yield as determined spectrophotometrically was 70%, using the value of 11,300 for ϵ_{max} at 260 mμ.

Pyridinium 2′-O-Acetyl-5′-O-monomethoxytrityluridine-3′ Phosphate.—A mixture of pyridinium 5′-O-monomethoxytrityluridine-3′ phosphate (0.1 mmole) and tetraethylammonium acetate (1.0 mmole) was rendered anhydrous by repeated evaporation of 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 at room temperature for 2 hr. Methyl alcohol (5 ml.) was then added and after 15 min. at room temperature the solution evaporated. To the residue were added water (5 ml.), ethyl alcohol (5 ml.), and pyridine (5 ml.) and the solution kept at room temperature for 2 hr. The solution was then passed through a column of pyridinium Dowex-50 ion exchange resin (bed volume 10 ml.) and the column washed thoroughly with 50% aqueous pyridine. The total effluent and washings were evaporated to a small volume, and after re-evaporation with dry pyridine the concentrated pyridine solution (5 ml.) was added dropwise to an excess of ether (150 ml.) under agitation. The resulting powder (66 mg.) was collected by centrifugation and washed with dry ether. It was stored over phosphorus pentoxide in a desiccator. Removal of the methoxytrityl group by treatment with aqueous Dowex-50 (H^+) resin at room temperature gave 2′-O-acetyluridine-3′ phosphate which, in turn, on ammoniacal treatment gave uridine-3′ phosphate. The yield of pure pyridinium 2′-O-acetyl-5′-O-monomethoxytrityluridine-3′ phosphate thus prepared was 92%.

2′-O-Acetyluridylyl-(3′ → 5′)-N,2′-O,3′-O-tribenzoyluridine (IV).—A mixture of pyridinium 2′-O-acetyl-5′-O-monomethoxytrityluridine-3′ phosphate (0.3 mmole) and N,2′-O,3′-O-tribenzoyluridine (0.9 mmole) was rendered anhydrous by repeated evaporation of their solution in dry pyridine. To the residual gum was added dry pyridine (2 ml.) followed by dry pyridinium Dowex-50 ion exchange resin (about 100 mg.) and DCC (300 mg., 1.45 mmoles) and the sealed reaction mixture kept at room temperature for 3 days. Water (2 ml.) followed by pyridine (4 ml.) was then added and after 4 hr. at room temperature the insoluble dicyclohexylurea was removed by filtration. The aqueous pyridine filtrate was extracted with petroleum ether and then evaporated under reduced pressure, pyridine being added during concentration. The residual gum was dissolved in pyridine (5 ml.) and the solution added dropwise to an excess of ether (250 ml.). The precipitate which formed was collected by centrifugation and dried in a desiccator. The dry powder was taken up in 50% aqueous ethyl alcohol (4 ml.), and Dowex-50 (H^+) ion exchange resin[20] was added gradually under agitation until the pH fell to 2.5. The solution was kept at this pH for 3 hr. at room temperature after which time some pyridine was added and the solution filtered from the resin, the latter being washed with 50% aqueous pyridine. The combined filtrate and washings were applied to the top of a DEAE–cellulose (carbonate form) column (45 × 3.5 cm.) in a 2° room. The column was washed with aqueous ethyl alcohol (20% in ethyl alcohol, 100 ml.) and then eluted with a linear gradient of triethylammonium bicarbonate. The reservoir contained 4 l. of 0.5 *M* triethylammonium bicarbonate in 20% ethyl alcohol, while the mixing vessel contained an equal volume of 20% aqueous ethyl alcohol. About 15-ml. fractions were collected at an approximate flow rate of 1 ml./min. The first ultraviolet-absorbing peak appeared in fractions 100–120 and it contained triethylammonium 2′-O-acetyluridine-3′ phosphate. The second peak (fractions 140–163) contained the symmetrical P^1,P^2-(2′-O-acetyluridine-3′) pyrophosphate whose characterization is described below. The desired product, 2′-O-acetyluridylyl-(3′ → 5′)-N,2′-O,3′-O-tribenzoyluridine, appeared in the third peak (fractions 180–500). These fractions were pooled and evaporated at 10° (Dry Ice–ethanol trap) under reduced pressure, pyridine being frequently added and the evaporation being repeated to remove triethylammonium bicarbonate completely. The final residue was taken up in pyridine (10 ml.) and the solution added dropwise to 250 ml. of anhydrous ether. The fine white precipitate was collected by centrifugation and dried in a desiccator at room temperature over phosphorus pentoxide. The yield, 230 mg., corresponded to 78%. This product traveled as a single spot on paper chromatography in solvent C; the R_f is given in Table I. The compound was completely resistant to the action of pancreatic ribonuclease.

The formation of some uridylyl-(3′ → 5′)-N,2′,3′-tribenzoyluridine (R_f in Table I) was detected when the chromatographic separation and isolation was not carried out at low temperature. The loss of the 2′-O-acetyl group conferred susceptibility toward pancreatic ribonuclease, the products being uridine-3′ phosphate and N,2′,3′-tribenzoyluridine.

Uridylyl-(3′ → 5′)-uridylyl-(3′ → 5′)-uridine.—Pyridinium 2′-O-acetyluridylyl-(3′ → 5′)-N,2′-O,3′-O-tribenzoyluridine (0.03 mmole) and pyridinium 2′,5′-di-O-acetyluridine-3′ phosphate (0.120 mmole) were rendered anhydrous together by repeated evaporation of their solution in dry pyridine. Finally, dry pyridine (0.5 ml.), dry pyridinium Dowex-50 ion exchange resin (about 100 mg.), and DCC (150 mg.) were added and the sealed reaction mixture shaken mechanically at room temperature for 3 days. Water (1 ml.) and pyridine (2 ml.) were then added and the mixture extracted with cyclohexane to remove unreacted DCC. After keeping the aqueous pyridine solution at room temperature for a further period of 12 hr., dicyclohexylurea was removed by centrifugation and the clear solution evaporated to a gum. Ammonium hydroxide (about 9 *N*) was then added and the mixture shaken for 8 hr. at room temperature. The solution was then concentrated to a small volume and the total applied to strips of Whatman paper 3 MM and chromatographed in solvent A for 24 hr. The ultraviolet-absorbing bands detected in the order of increasing R_f were: band 1, uridylyl-(3′ → 5′)-uridylyl-(3′ → 5′)-uridine, 2 μmoles; band 2, uridine-2′(3′) phosphate; band 3, uridylyl-(3′ → 5′)-uridine; band 4, uridine-2′,3′ cyclic phosphate; band 5, benzoic acid (R_f 77). Elution of the bands and spectrophotometric determination showed the yield of the desired product to be 80% as based on the amount of uridylyl-(3′ → 5′)-uridine recovered. This product was homogeneous as judged by paper chromatography and paper electrophoresis (R_f's in Tables I and II). Degradation with pancreatic ribonuclease using 7 optical density units at 260 mμ (equivalent to 0.7 μmole of the mononucleotide) showed complete disappearance of the starting material. Uridine-3′ phosphate and uridine were the only products, their proportion being 2.08:1.

Adenylyl-(3′ → 5′)-uridylyl-(3′ → 5′)-uridine.—A mixture of pyridinium 2′-O-acetyluridylyl-(3′ → 5′)-N-2′,3′-tribenzoyluridine (0.030 mmole) and pyridinium N,2′,5′-triacetyladenosine-3′ phosphate (0.120 mmole) was rendered anhydrous by repeated evaporation of their solution in dry pyridine. To the anhydrous gum was added dry pyridine (0.5 ml.), followed by dry Dowex-50 ion exchange resin (pyridinium) and DCC (0.150 g.). The reaction was kept sealed at room temperature for 3 days and was then treated with water (1 ml.) and pyridine (2 ml.). After extraction with cyclohexane and removal of the insoluble dicyclohexylurea, the clear aqueous pyridine solution was evaporated to a gum under reduced pressure. Ammonium hydroxide (5 ml. of 9 *N* solution) was added and after shaking at room temperature for 8 hr., the solution was evaporated to a gum and the mixture separated by preparative paper chromatography in solvent A. The following ultraviolet bands mentioned in the order of increasing R_f's were detected: adenylyl-(3′ → 5′)-uridylyl-(3′ → 5′)-uridine (R_f in Table I), adenosine-3′ phosphate, uridylyl-(3′ → 5′)-uridine, adenosine-2′,3′ cyclic phosphate, and benzoic acid. The yield of the desired product (ApUpU) as determined spectrophotometrically from the concentrations of the eluted bands (those of ApUpU and the unreacted UpU) was ascertained to be 75%. The product was homogeneous on paper electrophoresis as well and was completely degraded on incubation with the *Lactobacillus acidophilus* R-26 phosphodiesterase to give adenosine-3′ phosphate, uridine-3′ phosphate, and uridine. The conditions used were as described under General Methods, 9.6 optical density units (260 mμ) of the product being used in the incubation mixture. The products were separated by paper chromatography in solvent B and the concentrations of the different products determined spectrophotometrically after elution: adenosine-3′ phosphate (3.51 optical density units, 260 mμ), uridine-3′ phosphate (2.52 optical density units, 260 mμ), and uridine (2.6 optical density units). The molar proportions thus found for these products were: 0.94:1:1.04.

(20) More ethyl alcohol may be added at this stage to redissolve the cloudiness which appears on the addition of the ion exchange resin.

2'-O-Acetyl-N-benzoyladenylyl-(3' → 5')-2'-O-acetyluridylyl-(3' → 5')-N,2',3'-tribenzoylidine (VIII).—Pyridinium 2'-O-acetyluridylyl-(3' → 5')-N,2',3'-tribenzoyluridine (0.1 mmole) and pyridinium N-benzoyl-2'-O-acetyl-5'-O-monomethoxytrityladenosine-3' phosphate (0.5 mmole) (see accompanying paper[11]) were rendered anhydrous by repeated evaporation of their solution in dry pyridine. To the anhydrous gum was added dry pyridine (2 ml.) followed by pyridinium Dowex-50 ion exchange resin (100 mg.) and DCC (2.5 mmoles). The reaction mixture was kept sealed at room temperature for 3 days. Water (1 ml.) and pyridine (2 ml.) were then added and the mixture kept at room temperature for 6 hr. Dicyclohexylurea was then removed by filtration and the unreacted DCC extracted with petroleum ether. The aqueous pyridine solution was coevaporated with pyridine to a small volume (10 ml.) and the pyridine solution added dropwise to an excess (250 ml.) of dry ether. The precipitate was collected by centrifugation and dried under vacuum over phosphorus pentoxide. The weight of the dry powder was 910 mg.[21]

The dry powder (100 mg.) was dissolved in 2 ml. of 50% aqueous ethyl alcohol and the apparent pH of this solution was adjusted to 2.5 by gradual addition of Dowex-50 (H^+) ion exchange resin. (The addition of about 1 ml. of ethyl alcohol was necessary after the addition of the resin to redissolve the insoluble material which separated.) The solution was maintained at pH 2.5 for 3 hr. at room temperature, then treated with pyridine and filtered from the resin, the latter being washed with aqueous ethyl alcohol. The total solution was placed on a DEAE-cellulose (carbonate) column (32 × 2 cm.) in a 2° room. The column was washed with water containing 20% ethyl alcohol (1 l.) and elution was carried out with a linear gradient of triethylammonium bicarbonate (pH 7.5), the reservoir containing 0.5 *M* triethylammonium bicarbonate in 20% ethyl alcohol (3 l.) and the mixing vessel containing water-20% ethyl alcohol (3 l.). About 15-ml. fractions were collected every 10 min. The first peak, a minor one which appeared in fractions 40-50, has not been identified. The second peak (fractions 51-68) corresponded to N-benzoyl-2'-O-acetyladenosine-3' phosphate. Third peak (fractions 75-105) corresponded to P^1,P^2-(2'-O-acetyl-N-benzoyladenosine-3') pyrophosphate (X, R = N-benzoyladenine). The fourth peak (fractions 113-140, 117 optical density units at 260 mμ) contained 2'-O-acetyluridylyl-(3' → 5')-N,2',3'-tribenzoyluridine (IV) and the last peak (fractions 160-260) contained 2'-O-acetyl-N-benzoyladenylyl-(3' → 5')-2'-O-acetyluridylyl-(3' → 5')-N,2',3'-tribenzoyluridine (VIII) (396 optical density units). The yield of the desired product thus was about 72% as based on 2'-O-acetyluridylyl-(3' → 5')-N,2',3'-tribenzoyluridine used.

P^1,P^2-(2'-O-Acetyluridine-3') pyrophosphate (X, R = uracil) had R_f 0.78 in solvent B. Treatment with 9 *N* ammonium hydroxide at room temperature gave a mixture of uridine-2',3' cyclic phosphate and uridine-3' phosphate as judged by chromatography in solvent A. The decomposition to these products was found to be complete in the first aliquot (after 2 hr.) applied on paper chromatogram in solvent A.

Uridylyl-(3' → 5')-adenylyl-(3' → 5')-uridylyl-(3' → 5')-uridine (IX).—Pyridinium 2'-O-acetyl-N-benzoyladenylyl-(3' → 5')-2'-O-acetyluridylyl-(3' → 5')-N,2',3'-tribenzoyluridine (350 optical density units, about 10 μmoles) and pyridinium 2',3'-di-O-acetyluridine-3' phosphate (0.08 mmole) were rendered anhydrous by repeated evaporation of their solution in dry pyridine. To the anhydrous gum was added dry pyridine (0.5 ml.), pyridinium Dowex-50 ion exchange resin (100 mg.), and DCC (150 mg.) and the sealed reaction mixture kept for 3 days at room temperature. Water (1 ml.) and pyridine (2 ml.) were then added and after a further 15 hr. at room temperature dicyclohexylurea was removed by filtration and any unreacted DCC extracted with petroleum ether. The total aqueous pyridine solution was evaporated and the residue kept in 9 *N* ammonium hydroxide at room temperature for 24 hr. After removal of the solvent, one-fifth of the total products was placed on a DEAE-cellulose (carbonate) column (37 × 2 cm.) which was washed first with water (500 ml.) and then eluted with a linear gradient of triethylammonium bicarbonate (pH 7.5). The reservoir contained 3 l. of 0.4 *M* triethylammonium bicarbonate and the mixing vessel an equal volume of water. The first peak (fractions 90-110) contained uridine-3' phosphate. Adenylyl-(3' → 5')-uridylyl-(3' → 5')-uridine (12 optical density units at 260 mμ) appeared in fractions 131-145 and the last peak (fractions 210-235) contained the desired uridylyl-(3' → 5')-adenylyl-(3' → 5')-uridylyl-(3' → 5')-uridine (45 optical density units at 260 mμ). The yield as based on adenylyl-uridylyl-uridine recovered was 75%. The R_f's of the product are shown in Tables I and II. Degradation with the *Lactobacillus acidophilus* phosphodiesterase under the standard conditions using 8.5 optical density units of the product showed complete disappearance of the starting material. The resulting products as separated by paper chromatography in solvent B were adenosine-3' phosphate, uridine-3' phosphate, and uridine. Their concentrations in optical density units as determined after elution were: 1.53:2.2:1. The molar proportions of these products thus found were: 1.02:2.2:1.

(21) The total weight is in excess of the theoretical nucleotidic material used in the reaction mixture. It is certain that residual dicyclohexylurea is responsible for the excess weight. The total recovery of ultraviolet-absorbing material from the 100-mg. portion work-up described checks with the total nucleotidic material expected to be present.

[CONTRIBUTION FROM THE INSTITUTE FOR ENZYME RESEARCH, THE UNIVERSITY OF WISCONSIN, MADISON 6, WIS.]

Studies on Polynucleotides. XXIX.[1] The Specific Synthesis of $C_{3'}-C_{5'}$-Linked Ribooligonucleotides(5).[2] Homologous Adenine Oligonucleotides[3]

BY Y. LAPIDOT AND H. G. KHORANA

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The reaction of pyridinium adenosine-3' phosphate with mono- and di-*p*-methoxytrityl chlorides gave the corresponding 5'-O-methoxytrityl derivatives in good yields. Pyridinium adenosine-3' phosphate with an excess of benzoic anhydride at 50° in the presence of tetraethylammonium benzoate gave after work-up inclusive of an acetic anhydride-pyridine treatment N,2',5'-tribenzoyladenosine-3' phosphate. Alkaline treatment of the latter gave N-benzoyladenosine-3' phosphate. Reaction of the latter with monomethoxytrityl chloride followed by column chromatography gave 5'-O-monomethoxytrityl-N-benzoyladenosine-3' phosphate. Acetylation of the latter by the procedure previously developed followed by an acidic treatment gave N-benzoyl-2'-O-acetyladenosine-3' phosphate. Treatment of a mixture of the latter compound and pyridinium N,2',5'-triacetyladenosine-3' phosphate in dry pyridine with dicyclohexylcarbodiimide followed by successive acetic anhydride-pyridine and ammoniacal treatments gave homologous adenine oligonucleotides. Members up to the pentanucleotide, ApApApApAp, were isolated pure and fully characterized. The presence of only $C_{3'}-C_{5'}$ interribonucleotidic linkages in the products was demonstrated.

Methods for the chemical polymerization of deoxyribonucleotides and for the separation and characterization of the resulting homologous deoxyribopolynucleotides have been reported in a number of previous publications from this Laboratory.[4-6] Similar work in the ribopolynucleotide field was not undertaken[7] until

(1) Paper XXVIII: Y. Lapidot and H. G. Khorana, *J. Am. Chem. Soc.*, **85**, 3852 (1963).

(2) The previous papers which deal directly with the present topic: (a) ref. 1; (b) M. Smith, D. H. Rammler, I. H. Goldberg, and H. G. Khorana, *J. Am. Chem. Soc.*, **84**, 430 (1962); (c) D. H. Rammler and H. G. Khorana, *ibid.*, **84**, 3112 (1962); (d) D. H. Rammler, Y. Lapidot, and H. G. Khorana, *ibid.*, **85**, 1989 (1963).

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(6) R. K. Ralph and H. G. Khorana, *ibid.*, **83**, 2926 (1961).