Studies on the Specific Synthesis of the Natural Internucleotide Linkage by the Use of Cyclonucleosides. I. The Utilization of Unprotected Nucleotides*

Joseph Nagyváry†

ABSTRACT: The reaction of an excess of 2′,3′-O-isopropylidene-O^2,5′-cyclouridine with the monoanionic form of *p*-nitrophenyl phosphate, uridine 5′-phosphate, and thymidine 3′-phosphate at and over 100° gave a mixture of the mono- and di-2′,3′-O-isopropylideneuridine 5′-esters of these phosphates. This reaction represents the first direct synthesis of trinucleoside monophosphates. After mild acidic hydrolysis of the above mixtures triuridine 5′-phosphate, thymidine-3′-diuridine 5′-phosphate, and *p*-nitrophenyl diuridine 5′-phosphate were isolated in good yields. When uridine 3′-phosphate was heated in the same temperature range with an excess of 2′,3′-O-isopropylidene-O^2,5′-cyclouridine, no triester but uridylyl[2′(3′)→5′](2′,3′-O-isopropylidene)uridine was formed. This finding is explained by assuming the intermediate formation of a labile triester, 2′,3′-O-isopropylideneuridine-5′-uridine 2′,3′-cyclic phosphate, which is hydrolyzed to a 1:1 mixture of the isomeric diesters. At 75° the reaction of phosphodiesters with the cyclonucleoside is negligible and so diesters are the only products. Accordingly, at this temperature over 95% pure uridylyl(3′→5′)(2′,3′-O-isopropylidene)uridine was obtained in what appears to be the first chemical synthesis which produces mainly the natural internucleotide bond while utilizing an unprotected 3′-ribonucleotide.

Current chemical syntheses of the natural internucleotide bond are based upon activation of the phosphate group and full protection of all other reactive groups with the exception of one hydroxyl in the 3′- or 5′-position (Michelson, 1963). Considerable success has been achieved in this manner in the stepwise synthesis of deoxyribooligonucleotides (Jacob and Khorana, 1965) and ribooligonucleotides (Söll and Khorana, 1965; Chladek and Smrt, 1964; Scheit and Cramer, 1964; Griffin and Reese, 1964).

An alternative approach to dinucleoside phosphate synthesis is the provision of the 5′- or 3′-carbon of the nucleoside with a proper leaving group, followed by a nucleophilic displacement of this group by a nucleoside phosphate ion. Elmore and Todd (1952) demonstrated this principle in their synthesis of adenylyl(5′→5′)-uridine; they discontinued the use of this reaction later because of the low yield of the desired product due to a concomitant intramolecular displacement reaction leading to an O^2,5′-cyclonucleoside. The acid-catalyzed cleavage of isourea ethers of simple alcohols by phosphate esters was used by Khorana (1954) for the preparation of triesters even though the 5′-isourea ether of 2′,3′-O-isopropylideneadenosine also underwent cyclonucleoside formation (Khorana, 1961). It is apparent that the O^2,5′- and O^2,3′-cyclonucleosides of the pyrimidine bases contain the 5′- or 3′-carbon, respectively, in an isourea ether type grouping, and so they are expected to react with phosphates on thermal activation.

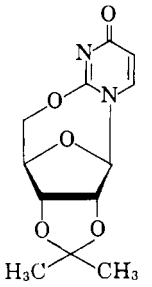
An increasing amount of work on pyrimidine cyclonucleosides has disclosed much valuable information about their reactivity toward a series of nucleophilic agents (Michelson, 1963, p 15; Brown *et al.*, 1957; Chambers and Kurkov, 1963; Miller and Fox, 1964). However, the utilization of the biologically most important nucleophile, the phosphate, in this reaction was started only very recently.

A synthesis of uridine 5′-phosphate by the reaction of a mixed phosphoric benzoic anhydride with 2′,3′-O-isopropylidene-O^2,5′-cyclouridine (I) was described by Mizuno *et al.* (1965).[1] Zemlicka and Smrt (1964) achieved the synthesis of the 3′→5′ internucleotide linkage in variable purity by the reaction of 2′,5′-O-diacetyluridine 3′-phosphate with 2′,3′-O-isopropylidene-O^2,5′-cyclouridine. When the free 3′-uridylate (dianion) was used, the production of the 2′→5′ and 3′→5′ isomers in equal amounts was observed, and hence a complete lack of specific bond formation. The similar though lower reactivity of the isomeric O^2,3′-cyclonucleoside was demonstrated most recently (Agarwal and Dhar, 1965).

* From the Institute of Cellular Biology, University of Connecticut, Storrs, Connecticut. *Received August 19, 1965; revised November 11, 1965 and January 20, 1966.* Supported by grants from the American Cancer Society (P-279) and the U. S. Public Health Service (GM 12732-01). A preliminary note on this subject appeared in *Tetrahedron Letters, No. 11,* 617 (1965).

† Present address: The Creighton University School of Medicine, Department of Biochemistry, Omaha, Neb.

[1] Their preliminary report in the Abstract of the 19th National Meeting of the Pharmaceutical Society of Japan, Tokyo, April, 1964, was not available to us.



I

As previously reported (Nagyváry and Roth, 1965; Nagyváry, 1965) our research has been proceeding independently with particular emphasis on the utilization of unprotected nucleotides and the extension of this method for oligonucleotide synthesis. This paper is concerned with the first part of this work.

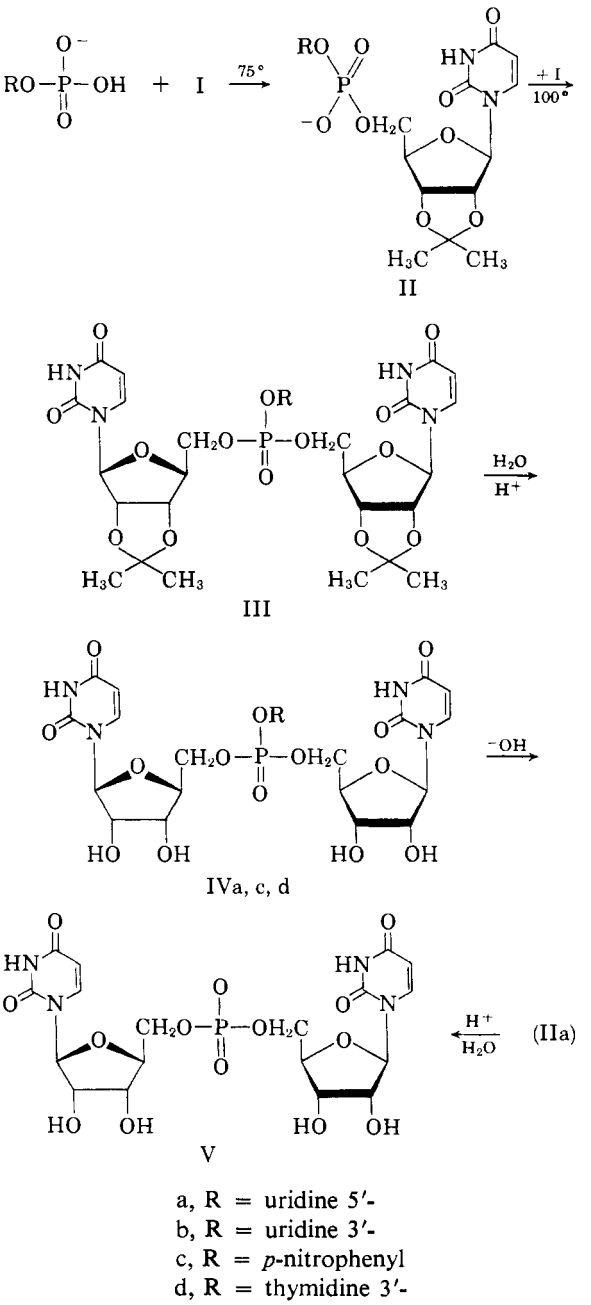
Results

The monoanionic form of the phosphates indicated below was heated in dimethylacetamide with the cyclonucleoside I at temperatures between 60 and 140° and with incubation times of from 3 hr to 1 week. The amount of cyclonucleoside varied from 2 to 4 equiv. Such excess is necessary when working on a small scale (0.05–0.1 mmole) because a variable portion of this component is hydrolyzed by the small amount of moisture still present in the system. For this reason, following the reaction by the disappearance of the cyclonucleoside has limited value.

It has been observed that on appropriate thermal activation, between 100 and 140°, the reaction of *p*-nitrophenyl phosphate, uridine 5′-phosphate, and thymidine 3′-phosphate with an excess of I yielded within 4–12 hr a mixture of the mono- and di-2′,3′-O-isopropylideneuridine 5′-esters of these nucleophiles (IIa–d, Scheme I). The formation of a phosphate triester in the presence of significant amounts (20–40%) of monoester was surprising and long overlooked. When the same mixtures were heated from 60 to 75°, phosphodiesters were produced as the only products. Similarly, no triesters were formed when 1 equiv of cyclonucleoside only was utilized at 100°; the yield of phosphodiester, however, was only about 20%. The yield of phosphodiester could not be raised over 35% by increasing the excess of cyclonucleoside due to the concomitant formation of the phosphotriester (Figure 1).

The great lability of *p*-nitrophenyl diuridine 5′-phosphate (IVc) in alkaline medium, in which the yellow *p*-nitrophenolate is instantaneously formed, led to the first observation of a triester in the nucleoside fraction. Thereafter also triuridine 5′-phosphate (IVa) could be detected and isolated by paper chromatography. The structures of these substances were deduced from the following observations: (1) Their chromatographic and electrophoretic properties are similar to those of nonionic compounds. (2) They are relatively stable in dilute acid, but are hydrolyzed in alkali to a mixture of diuridine 5′-phosphate (V) and *p*-nitrophenol, or uridine,

SCHEME I



a, R = uridine 5′-
b, R = uridine 3′-
c, R = *p*-nitrophenyl
d, R = thymidine 3′-

respectively. (3) Repeated determination of ϵ(P) (absorbance per gram-atom of P) gave the values 25,200 (IVc) and 29,000 (IVa), indicating the presence of two and three 1-alkyluracil moieties, respectively, per 1 phosphorus atom (uridine: ϵ_{261} 9700). (4) The composition of triuridine 5′-phosphate is also supported by a direct phosphorus analysis. (5) Both compounds are resistant to snake venom diesterase.

In addition to these two triesters, diuridine-5′-thymidine 3′-phosphate (IVd) and the corresponding isopropylidene derivative (IIId) were also isolated. For chromatographic values see Table I. The compound IVd was prepared with the intention to demonstrate the

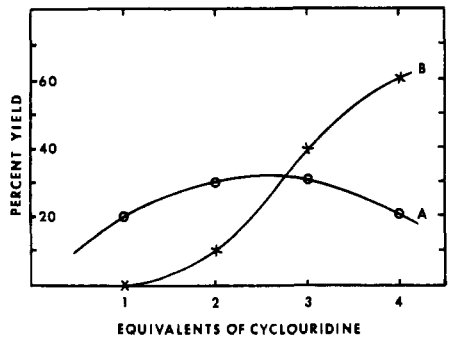


FIGURE 1: Reaction of uridine 5′-phosphate with cyclouridine (I) at 130°, 5 hr. (A) Yield of diester IIa; (B) yield of triester IIIa.

TABLE 1: R_F Values of Different Compounds on Paper Chromatograms.

	Solvent			
	A	B	C	D
Uridylyl(3′→5′)(2′,3′-O-isopropylidene)-uridine (IIb)	0.32		0.65	
Uridylyl(5′→5′)-uridine (V)	0.14			
Uridine 2′,3′-cyclic phosphate	0.24			
Uridine 5′-phosphate	0.08			
Uridine 3′-phosphate	0.07			
Di(2′,3′-O-isopropylideneuridine-5′)uridine 5′-phosphate (IIIa)	0.44	0.39	0.70	0.66
Triuridine 5′-phosphate (IVa)	0.08	0.01	0.26	0.27
Di(2′,3′-O-isopropylideneuridine-5′)thymidine 3′-phosphate (IIId)		0.45	0.74	0.66
Diuridine-5′-thymidine 3′-phosphate (IVd)		0.06	0.28	0.42
p-Nitrophenyl diuridine 5′-phosphate (IVc)		0.20		
Uridine	0.43	0.26	0.54	0.66
2′,3′-O-Isopropylideneuridine		0.75		
2′,3′-O-Isopropylidene-O^2,5′-cyclouridine (I)		0.65		

stability of a triester containing one deoxyribonucleoside 3′ linkage under the conditions of this reaction.

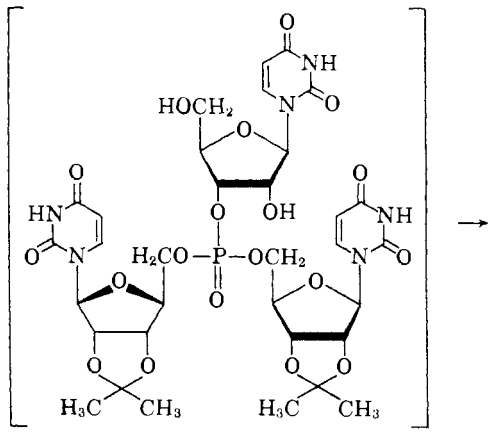
Specific Synthesis of the Natural Internucleotide Bond. The discovery of triester formation has special consequences for planning the reaction of unprotected ribonucleoside 3′-phosphate with cyclonucleosides. Zemlicka and Smrt (1964) found that uridine 3′-phosphate (dianion) reacts with the cyclonucleoside I at 100° to yield an equal mixture of the 2′→5′ and 3′→5′ diuridine phosphates, instead of forming the natural internucleotide linkage exclusively, as had been expected. This observation is in agreement with the results of our experiments with the monoanionic form of uridine 3′-phosphate.[2] An explanation for this isomerization is provided by the assumption of triester formation in analogy to our previous findings. As was demonstrated by Brown *et al.* (1955), the dimethyl and dibenzyl esters of uridine 3′-phosphate are unstable over the whole pH range. The instability of such phosphotriesters is explained by the participation of the vicinal hydroxyl group in the hydrolysis. According, the initially formed labile di-(2′,3′-O-isopropylideneuridine-5′)uridine 3′-phosphate (IIIb) should undergo hydrolysis to uridylyl[2′(3′)→5′]-(2′,3′-O-isopropylidene)uridine as outlined in Scheme II. A transesterification of IIIb leading to 2′,3′-O-isopropylideneuridine-5-uridine 2′,3′-cyclic phosphate (VI) might have already occurred in anhydrous medium prior to hydrolysis. Furthermore, Zemlicka and Smrt (1964) have described the partial isomerization of pure uridylyl-(3′→5′)uridine on treatment with 2′,3′-O-isopropylidene O^2,5′-cyclocytidine, and the isolation of uridylyl-[2′-(3′)→5′]-(2′,3′-O-isopropylidene)cytidine. This isomerization and exchange reaction may be regarded as supporting the view that there is an intermediate formation of a labile phosphotriester. On the other hand, it appears to be reasonable that the synthesis of pure 3′→5′ isomer may be possible only if this triester formation is successfully avoided.

As we found earlier, the formation of phosphodiesters can be favored over triesters by choosing a lower temperature. When the unprotected 3′-uridylate was allowed to react with 3–4 equiv of I at 75° for 1 week, a mixture of uridylyl(3′→5′)(2′,3′-O-isopropylidene)-uridine (IIb), with yields up to 24%, and uridine 2′,3′-cyclic phosphate (3–6%) were obtained. Over 95% of the first product was hydrolyzed by pancreatic ribonuclease to uridine 3′-phosphate and 2′,3′-O-isopropylideneuridine. Preliminary results have also shown that the failure to protect the 2′- and 3′-hydroxyl groups of the cyclonucleoside does not affect the purity of the internucleotide linkage. The progress of these studies is limited by our inability to obtain the O^2,5′-cyclouridine in acceptable yields and free from contaminating uridine.

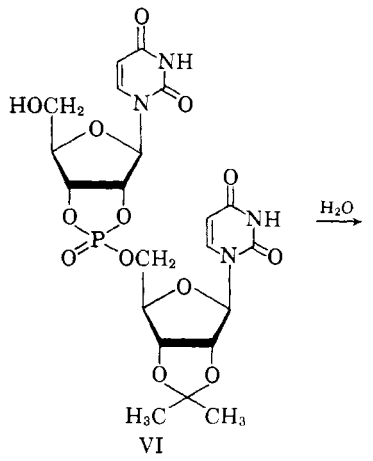
Possible Mechanism. Due consideration should be given also to the mechanism by which the new P–O–C(5′) bond is formed in the general reaction of pyrimidine O^2,5′-cyclonucleosides with phosphomonoesters as nucleophiles. The question is whether the new 5′-phosphate ester linkage is established by a direct attack of the phosphate on C-5′ of the cyclonucleoside, as desired, or through the intermediate attachment of phosphate on C-2 of the pyrimidine ring followed by

[2] We were not able to increase the yield of the dinucleoside phosphates over 50%. The yields of both Zemlicka and Smrt (1964) and Mizuno *et al.* (1965) were calculated from the amount of unchanged cyclonucleoside.

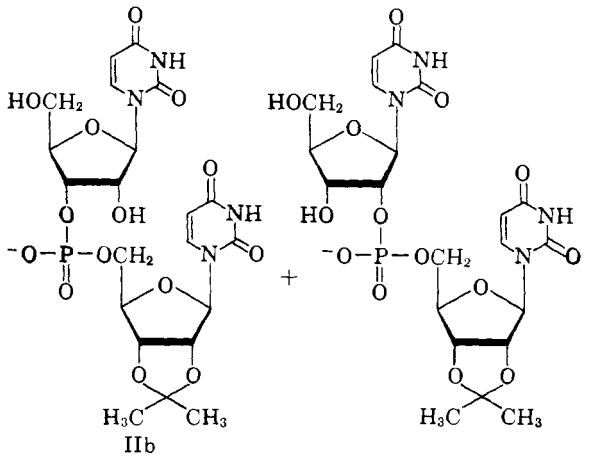
SCHEME II



IIIb



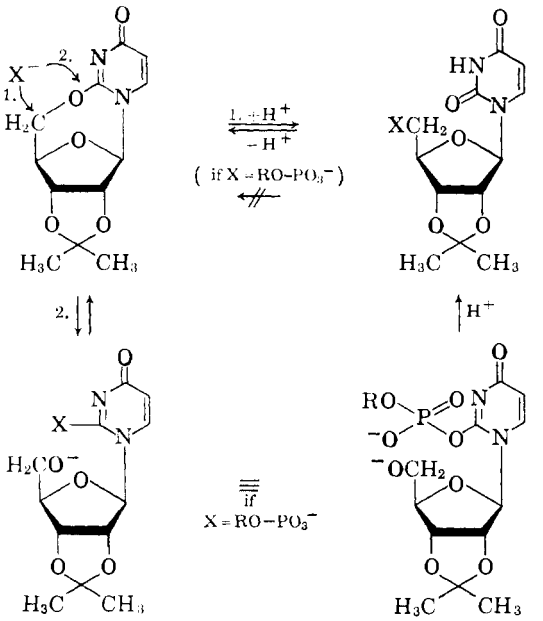
VI



IIb

migration to C-5′ (Scheme III). The latter case would represent a new intramolecular activation of the phosphomonoester group. The answer may be tentatively obtained after a study of available information on the reaction of various nucleophiles with cyclonucleosides (Brown *et al.*, 1957, 1958; Chambers and Kurkov, 1963; Miller and Fox, 1964; Codington *et al.*, 1964). In analogy to other nucleophiles of little basicity, such as HS^-, Br^- and I^-, the phosphates may attack either the carbon-2 of the pyrimidine ring or the carbon-5′ of the

SCHEME III



X^- = Cl^-, Br^-, I^-
p-toluenesulfonate, $ROPO_3^{2-}$

sugar with similar probability. Contrary to this, it was proved that the hydrogen ion catalyzed cleavage of the $O^2,3'$-cyclonucleoside ether linkage takes place at the ribose carbon under the inversion thereof (Fox and Miller, 1963; Miller and Fox, 1964; Codington *et al.*, 1964). As the monoanionic form of phosphate can produce at elevated temperature simultaneously its more nucleophilic conjugate base and H^+ ions, the direct formation of the 5′-diester linkage in our experiments is very likely. The greater steric accessibility of the primary carbon (5′) in I for nucleophilic attack in comparison with the isomeric nucleosides containing secondary (2′ or 3′) ether linkages gives further support to this hypothesis.

Conclusion

This work is of theoretical interest since it represents the first successful effort to utilize an unprotected ribonucleotide in the specific synthesis of the natural (3′→5′) internucleotide linkage. On the other hand, it is not likely that the phosphodiester formation *via* cyclonucleosides can be utilized in the practical synthesis of ribooligonucleotides without protecting the 2′-hydroxyl group. The success of both stepwise synthesis and polymerization requires the improvement of yields and the ready availability of phosphorylated cyclonucleosides. Work in this direction is advancing in our laboratory (Nagyváry, 1965).

The reaction of phosphodiesters with cyclonucleosides offers the intriguing possibility of introducing branching into the DNA chain. The ready formation of trinucleoside phosphates by means of this reaction is especially remarkable, because, as far as we are

aware, this class of compounds has not been prepared before by direct synthesis.[3] The preparation of trinucleoside monophosphates for biochemical studies can now also be undertaken.

Experimental Section

Materials

2′,3′-O-isopropylidene-O²,5′-cyclouridine (I) was obtained as described below by our modification of the general procedure of Michelson and Cohn (1962) in over 50% yield, based upon 2′,3′-O-isopropylideneuridine.

p-Nitrophenyl phosphate and uridine 5′-phosphate were purchased from a commercial source (Sigma). Thymidine 3′-phosphate was prepared according to Tener (1961). Uridine 3′-phosphate was obtained from uridine 2′(3′)-phosphate (Boehringer, or Schwartz) by recrystallizing the disodium salt from alcohol–water. Its purity was checked by ion-exchange chromatography (Cohn, 1957). These phosphates were stored as pyridinium salts at −10° in dimethylacetamide solution of known concentration.

The solvents were purified as follows: Dimethylacetamide (DMA) and *s*-collidine were distilled *in vacuo* and stored over molecular sieve (Linde, Type 4A). Pyridine and dioxane were first distilled over KOH, then refluxed 1 hr over phosphorus pentoxide and redistilled.

Methods

Descending paper chromatography for analytical purposes was carried out on Whatman No. 1 paper in the following solvents: 2-propanol–concentrated ammonia–water 7:1:2 (A), 1-butanol saturated with water (B), ethanol–0.5 M ammonium acetate (pH 7.2) 5:2 (C), and 1-butanol–acetic acid–water 5:2:3 (D).

Paper electrophoresis on Whatman No. 3MM paper was conducted in a Buchler apparatus (10 v/cm) with 0.05 M phosphate buffer pH 7.5. Monesters and diesters of phosphoric acid were easily characterized with the help of markers.

All isolation procedures were done by chromatography on Whatman No. 3MM paper sheets which had been previously washed at least 1 week in the solvent to be used. The spots or bands of the compounds were marked under ultraviolet light, cut into small pieces, packed into a small column, and eluted with 25 or 50 ml of water. The determination of yield or product ratios was accomplished by measuring the optical densities of the compounds in the form of ammonium salts at neutral pH at the wavelength of maximum absorption. The use of blanks proved unnecessary.

Phosphorus was determined according to King (1932). The ϵ(P) (Chargaff and Zamenhof, 1948) was experimentally determined as the quotient of the 1-cm absorbance at the spectral maximum by the phosphorus molarity.

All evaporations were carried out under reduced pressure at temperatures not exceeding 25°. Drying of the reaction mixture containing one of the phosphates and the cyclonucleoside occurred by repeated evaporation from DMA and *s*-collidine. To the concentrated solution (about 0.3 ml), which still contained some *s*-collidine, was added 1.1 equiv of tri-*n*-butylamine or tri-*n*-octylamine in 1 ml of the solvent employed, and the flask was sealed under vacuum.

An Improved Preparation of 2′,3′-O-Isopropylidene-O²,5′-cyclouridine (I). The general method of Michelson and Cohn (1962) for the preparation of O²,5′-cyclonucleosides is based upon the reaction of the corresponding tosylate with potassium *t*-butoxide. We found that the strong organic base, N,N′-dicyclohexyl-4-morpholinocarboxamidine, which was prepared according to Moffatt and Khorana (1961), can be used with considerable advantage. Its tosylate, the by-product of the reaction, shows good solubility in methanol–ether, and so it can be separated from the insoluble cyclonucleoside by repeated precipitation in anhydrous medium.

Amorphous 5′-*p*-tosyloxy-2′,3′-O-isopropylideneuridine was prepared according to Levene and Tipson (1934) and was crystallized from acetone–benzene. When dried at room temperature and 0.05 mm for 5 hr, the crystals retained 1 mole of benzene (mol wt 516.5; mp 146–147°). A slight excess of the tosylate (2.1 mmoles, 1.086 g) and the carboxamidine base (2.0 mmoles, 590 mg) were separately freeze-dried from dioxane; then they were dissolved and mixed together in 20 ml of dioxane containing about 10% of DMA. The sealed flask was warmed for 4 hr at 80° (or 18 hr at 45°); the solution was then dropped into 100 ml of dry ether. After 1 hr at 0° the precipitate was filtered off, dissolved in methanol, and precipitated again with an excess of ether. The white amorphous powder (505 mg) contained only a negligible amount of the organic salt, as was ascertained by ultraviolet absorption measurements at 220 and 240 mμ, and so it could be used in the following experiments. Crystallization from methanol–ether gave 432 mg (78%) of pure 2′,3′-O-isopropylidene-O²,5′-cyclouridine (I), which had the same ultraviolet and chromatographic values and decomposition point as reported by Brown *et al.* (1957).

The Reaction of Uridine 5′-Phosphate with Cyclouridine (I). A. AT HIGH TEMPERATURE. Monotri-*n*-butylammonium (or *s*-collidinium) uridine 5′-phosphate (0.063 mmole) was mixed with 1–4 equiv of cyclouridine (I) which was dissolved in 1 ml of DMA, and the mixtures heated at 130° for 5 hr in sealed tubes. In order to separate the phosphate anions from the uncharged material, the reaction mixtures were diluted with acetone–water (1:1) and each was passed through a 1 × 20 cm Dowex 1-X8 (formate) column. Each column was washed with 100 ml of acetone–water and the total eluates were concentrated. Each concentrate was applied to three 18-cm wide Whatman No. 3MM

[3] Evidence for the formation of some *t*-phosphate ester linkages in course of the synthesis of deoxyribooligonucleotides was earlier presented by Weimann and Khorana (1962), who identified the end product of enzymatic degradations as dithymidine-5′-thymidine 3′-phosphate.

paper strips which then were developed in solvent B. A test for phosphate with the Hanes–Isherwood reagent (1949) gave a negative result. Bands at R_F 0.75 and 0.62 corresponding to 2′,3′-O-isopropylideneuridine and cyclouridine (I) were discarded. The bands at R_F 0.40, which were present in increasing width in expt 2–4, were eluted with butanol–water. The amounts of this substance, which represents the triester IIIa, were determined by measuring the ultraviolet absorption at 261 mμ and were found to be 180, 730, and 1090 ODU in expt 2–4, respectively (1 mmole of uridine corresponds to 9700 ODU). The nucleotides which were absorbed on the ion-exchange columns were resolved into IIa and uridine 6′-phosphate by elution with a linear gradient (0.01–0.15 M) of ammonium formate pH 7.5. After repeated freeze-drying the amounts of uridylyl(5′→5′)-(2′,3′-O-isopropylidene)uridine (IIa) in expt 1–4 were determined to be 250, 370, 380, and 260 ODU. The yields of products in this reaction are depicted in Figure 1. Recoveries were between 80 and 90%.

B. AT 75°. Four tubes of the same content as above were kept at 75° for 1 week. Paper chromatograms developed in solvent B showed the absence of triester IIIa in all cases. The diester IIa was isolated by means of paper chromatography in solvent A. The yields were 3, 13, 22, and 32%, respectively, as determined spectrophotometrically.

Triuridine 5′-Phosphate (IVa). One portion of the nonionic compound IIIa was freeze-dried from water and dried at 80° and 0.02 mm for 5 hr over phosphorus pentoxide. *Anal.* Calcd for the water-free formula $C_{30}H_{41}N_6O_{19}P$ (820.6) P: 3.8%. Found: 3.4%. The lower value suggests the presence of water or some contamination, possibly from paper. Efforts to crystallize the compound were without success.

Under acidic conditions, *e.g.*, under reflux in 80% acetic acid for 60 min or in 0.1 N HCl at room temperature for 1 day, the protecting groups can be eliminated and triuridine 5′-phosphate (IVa) is formed. For chromatographic values see Table I. Neither IIIa nor IVa migrates on electrophoresis at neutral pH. The unprotected triester exhibited in two determinations of ε(P) the values 28,100 and 30,000, well in accord with the presence of three uridine moieties (ϵ_{261} 9,700) in the molecule.

A further support for the formula IVa for the nonionic compound was obtained from the results of alkaline hydrolysis. In a qualitative experiment 10 OD units of the triester was heated in excess of 0.1 N KOH at 80° for 4 hr. Electrophoretic analysis of the mixture at pH 7.5 indicated partial hydrolysis to a diester; traces of uridine 5′-phosphate were also detected.

Attempts to enzymatically hydrolyze the triester IVa: 10 ODU of this material was incubated with 20 μg of purified snake venom diesterase (Worthington) in 0.05 ml of 0.1 M Tris buffer, pH 8, at 37° for 6 hr. No change was observed on the paper chromatogram after this time. Under the same conditions, the diester was completely degraded to uridine 5′-phosphate and uridine. Its stability in dilute KOH at 40° during 24 hr combined with the previous findings sufficiently identified this compound as uridylyl(5′→5′)uridine (V).

p-Nitrophenyl Diuridine 5′-Phosphate (IVc). Collidinium *p*-nitrophenyl phosphate (0.05 mmole) was treated with 42 mg (0.15 mmole) of cyclouridine in 1 ml of DMA at 110° for 10 hr. After acidic cleavage of the isopropylidene groups, the isolation followed the previously applied combination of ion-exchange and paper chromatography. The yield was 30%. *p*-Nitrophenyl diuridine 5′-phosphate (IVc) did not migrate on electrophoresis in 0.05 M ammonium acetate buffer pH 6. Its paper chromatographic mobility in solvent B relative to uridine (R_U) is 0.80. At the absorption maximum (263 mμ), ε(P) = 25,200. Dissolving 6.67 ODU of IVc in 10 ml of 0.1 N KOH gave after 20 min at room temperature the absorbancy reading A = 0.430 at 400 mμ; calculated A = 0.480. The hydrolysis to *p*-nitrophenol and uridylyl(5′→5′)uridine (V) was quantitative according to an electrophoretic comparison. Also this triester was stable to purified snake venom diesterase.

Thymidine-3′-diuridine 5′-Phosphate (IVd). Collidinium thymidine 3′-phosphate (0.05 mmole) and 42 mg (0.15 mmole) of cyclouridine in 1 ml of DMA were heated at 130° for 5 hr. The isolation of thymidine-3′-di-(2′,3′-O-isopropylideneuridine-5′)phosphate (IIId) and its acidic hydrolysis to IVd were carried out as described for IIIa. The yield for IIId was 45%. For paper chromatographic values see Table I.

The Reaction of Uridine 3′-Phosphate with Cyclouridine (I). A. AT 70–75°. SYNTHESIS OF URIDYLYL-(3′→5′)(2′,3′-O-ISOPROPYLIDENE)URIDINE (IIB). To 0.1 mmole of monotri-*n*-octylammonium uridine 3′-phosphate was added 107 mg (0.4 mmole) of cyclouridine (I) in 2 ml of acetonitrile–DMA (or dioxane–DMA) 9:1. The sealed flask was kept at temperatures between 70 and 75° for 1 week. After exchange of the cation to NH_4^+ on a small Dowex 50 column, the concentrated solution was applied to three 25-cm wide Whatman No. 3MM paper strips. Solvent B was allowed to run beyond the length of the 45-cm paper in order to separate the remaining cyclouridine 2′,3′-O-isopropylideneuridine and traces of uridine far enough from the nucleotidic material. After drying, the same sheets were developed with solvent A for 24 hr, by which time the separation of the four bands containing phosphorus was satisfactory. The elution was carried out with water, and four fractions were obtained.

Fraction 1 consists of 382 ODU of uridylyl(3′→5′)-(2′,3′-O-isopropylidene)uridine (IIb); yield 19%. Its chromatographic values are given in Table I. This substance (10 ODU) and 20 μg of crystalline ribonuclease (RNAase) (Worthington) were incubated in 0.05 ml of 0.1 M Tris–chloride buffer, pH 8.0, at 37° for 2 hr. A direct examination of the mixture by paper chromatography in solvents A and B and by electrophoresis showed that about 96% of IIb was degraded to a 1:1 mixture of uridine 3′-phosphate and 2′,3′-O-isopropylideneuridine.

Fraction 2 (23 ODU) was completely hydrolyzed by RNAase to uridine 3′-phosphate. In chromatogram and electrophoresis it was identical with uridine 2′,3′-

cyclic phosphate. The ratio of this substance showed significant variation in parallel experiments. Fraction 3 (62 ODU) was not identified. It was not entirely separated from fraction 4 (508 ODU) which contained the unreacted monoester.

The lowest temperature at which the formation of the diester was still observed was 60°; the yield, however, was less than 10%.

B. Reaction at 130°. Synthesis of uridylyl[2′-(3′)→5′](2′,3′-O-isopropylidene)uridine. Monotri-*n*-octylammonium 3′-uridylate (0.05 mmole) and 52 mg of cyclouridine (I) were dissolved in 1 ml of dioxane–DMA (9:1), sealed *in vacuo*, and heated at 130° for 5 hr. The products were isolated as described above. The best yield of the dinucleoside phosphate fraction was 50%. An aliquot of this was half degraded by pancreatic RNAase, which indicates that the mixture consists of equal amounts of the two isomers.

Acknowledgment

The author is deeply indebted to the Swiss "Stiftung für Stipendien auf dem Gebiete der Chemie" for a postdoctoral fellowship at Cambridge University, England, where this work was initiated. He is grateful to Lord Todd for the encouragement and helpful suggestions on the manuscript. Thanks are also due to Mrs. Eva Nagyváry for skillful technical assistance.

References

Agarwal, K. L., and Dhar, M. M. (1965), *Tetrahedron Letters 29*, 2451.

Brown, D. M., Magrath, D. I., and Todd, A. R. (1955), *J. Chem. Soc.*, 4396.

Brown, D. M., Parihar, D. B., and Todd, A. R. (1958), *J. Chem. Soc.*, 4242.

Brown, D. M., Todd, A. R., and Varadarajan, S. (1957), *J. Chem. Soc.*, 868.

Chambers, R. W., and Kurkov, V. (1963), *J. Am. Chem. Soc. 85*, 2160.

Chargaff, E., and Zamenhof, S. (1948), *J. Biol. Chem. 173*, 327.

Chladek, S., and Smrt, J. (1964), *Collection Czech. Chem. Commun. 29*, 214.

Codington, J. F., Doerr, I. L., and Fox, J. J. (1964), *J. Org. Chem. 29*, 564.

Cohn, W. E. (1957), *Biochem. Prepn. 5*, 40.

Elmore, D. T., and Todd, A. R. (1952), *J. Chem. Soc.*, 3681.

Fox, J. J., and Miller, N. (1963), *J. Org. Chem. 28*, 936.

Griffin, B. E., and Reese, C. B. (1964), *Tetrahedron Letters 40*, 2925.

Gulland, J. M., and Smith, H. (1948), *J. Chem. Soc.*, 1532.

Hanes, C. S., and Isherwood, F. R. (1949), *Nature 164*, 1107.

Jacob, T. M., and Khorana, H. G. (1965), *J. Am. Chem. Soc. 87*, 368.

Khorana, H. G. (1954), *Can. J. Chem. 32*, 227.

Khorana, H. G. (1961), Some Recent Developments in the Chemistry of Phosphate Esters of Biological Interest, New York, N. Y., Wiley, p 102.

King, E. J. (1932), *Biochem. J. 26*, 292.

Levene, P. A., and Tipson, R. S. (1934), *J. Biol. Chem. 106*, 113.

Michelson, A. M. (1963), The Chemistry of Nucleosides and Nucleotides, New York, N. Y., Academic, Chapter 7.

Michelson, A. M., and Cohn, W. E. (1962), *Biochemistry 1*, 490.

Miller, N., and Fox, J. J. (1964), *J. Org. Chem. 29*, 1772.

Mizuno, Y., Sasaki, T., Kanai, T., and Igarashi, H. (1965), *J. Org. Chem. 30*, 1533.

Moffatt, J. G., and Khorana, H. G. (1961), *J. Am. Chem. Soc. 82*, 649.

Nagyváry, J. (1965), Abstracts, 150th National Meeting of the American Chemical Society, Atlantic City, N. J., Sept 1965, p 87C.

Nagyváry, J., and Roth, J. S. (1965), *Tetrahedron Letters 11*, 617.

Scheit, K. H., and Cramer, F. (1964), *Tetrahedron Letters 38*, 2765.

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

Tener, G. M. (1961), *J. Am. Chem. Soc. 83*, 159.

Weimann, G., and Khorana, H. G. (1962), *J. Am. Chem. Soc. 84*, 419.

Zemlicka, J., and Smrt, J. (1964), *Tetrahedron Letters 31*, 2081.