Arabinonucleotides. I. A Study of the Formation of 3′-Arabinonucleotidyl Esters by the Conversion of an Internucleotide Linkage*

Joseph Nagyvary and Roberto G. Provenzale

ABSTRACT: A novel reaction of ribooligonucleotides involves the conversion of the ribose moiety of the pyrimidine nucleotides into derivatives of β-D-arabinose without breaking the 3′–5′-internucleotide bonds. The reaction consists of three steps: (1) an activation which generates 2′:3′→5′-cyclic triesters, (2) a thermal rearrangement which produces 3′–5′-linked diesters containing O^2:2′-cyclonucleosides, and (3) mild alkaline hydrolysis leading to the formation of arabinooligonucleotides. This rather complex process has now been extensively studied on the simplest model, a suitably protected 2′(3′)→5′-linked diuridine monophosphate. The cyclic triester intermediate, 5′-*O*-acetyluridylyl-[(2′:3′)→5′]-(2′:3′-*O*-isopropylidene)uridine, was also obtained by a *de novo* synthesis by the general method of Michelson (1959). The formation of 5′-*O*-acetyl-O^2:2′-cyclouridylyl-(3′→5′)-(2′:3′-*O*-isopropylidene)uridine was found to depend on the nature and concentration of the activating agent, the solvent, the temperature, and is base-catalyzed. This step could be at least partially reversed by anhydrous acid.

The irreversible alkaline hydrolysis, followed by acidic deblocking, gave arauridylyl-(3′→5′)-uridine in virtually quantitative yield. These results provide useful information with regard to the synthesis and chemistry of polyarabinonucleotides.

Attempts to prepare the arabino analogs of polynucleotides enzymatically have been unsuccessful so far (Michelson *et al.*, 1962). To the organic chemist, the failure of the polynucleotide–phosphorylase system represents a challenge which is further stimulated by the known biological activity of the arabinonucleotides and dinucleotides (Cohen, 1966; Wechter, 1967; Renis *et al.*, 1967). The numerous difficulties involved in linking the arabinonucleosides with 3′→5′-phosphodiester linkages according to the classical scheme, together with the scarcity of the starting materials, have provided the incentive to search for unorthodox approaches.

A most unconventional idea in biopolymer synthesis, which originated in Schramm's laboratory and is based on the use of substituted polyphosphates as solvent and activating agent, has recently led to the identification of "polyspongouridylate," *i.e.*, polyarauridylate as one of the reaction products (Schramm and Ulmer-Schurnbrand, 1967). This simple method, however, seems to be somewhat limited as far as the medium chain length and the yield of polymers are concerned.

The basic concept underlying our own efforts of polyarabinonucleotide synthesis is the conversion of the ribo moiety into the arabino moiety at the oligonucleotide level. The theoretical feasibility of such a conversion was provided a decade ago by Dekker's group (Walwick *et al.*, 1959) through their synthesis of arabinonucleosides from ribonucleosides in polyphosphoric acid. If one assumes that 2′:3′-cyclic pyrophosphates were the reactive intermediates, as it was suggested by Walwick (1958), then one can expect a similar reactivity of dinucleoside (2′:3′)→5′ cyclic triesters. And indeed, in a preliminary form (Nagyvary, 1967a,b) we were able to report the thermal rearrangement of the cyclic triesters of uridine and cytidine, in which diesters containing O^2:2′-cyclonucleosides were formed. These intermediates could be hydrolyzed to arabinonucleotides. This idea is outlined in Scheme I. Thus a rational approach was discovered for a very simple synthesis of polyarabinonucleotides of the pyrimidine bases, capable of producing a relatively high molecular weight polymer in good yield.

Some properties of polyarauridylic acid have already been published (Schramm and Ulmer-Schurnbrand, 1967; Nagyvary *et al.*, 1968), the most outstanding of which is its inability to function as a messenger. It was also suggested that these polymers could have formed a starting point in the prebiotic evolution of polynucleotides (Nagyvary, 1968).

The observation of varying amounts of by-products in our polymeric mixtures has necessitated a thorough investigation on a simpler system. This paper contains a comparative evaluation of several possible formations and rearrangements of cyclic triesters, using diuridine phosphate as a model. Because of its simplicity, this system is also suitable for establishing and studying the important factors that determine the kinetics and degree of rearrangement.

Experimental Procedures

Materials

5′-O,N^3-Diacetyluridine 2′:3′-cyclic phosphate is expected to be the primary product in the acetylation of uridine 2′:3′-cyclic phosphate in dioxane and tri-*n*-butylamine according

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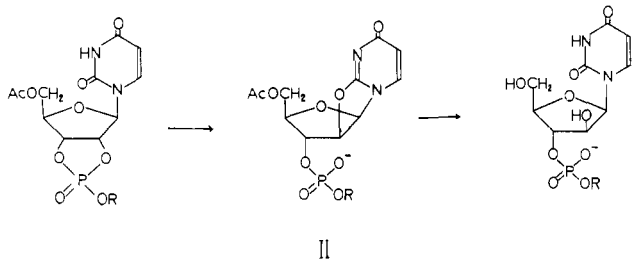
SCHEME I



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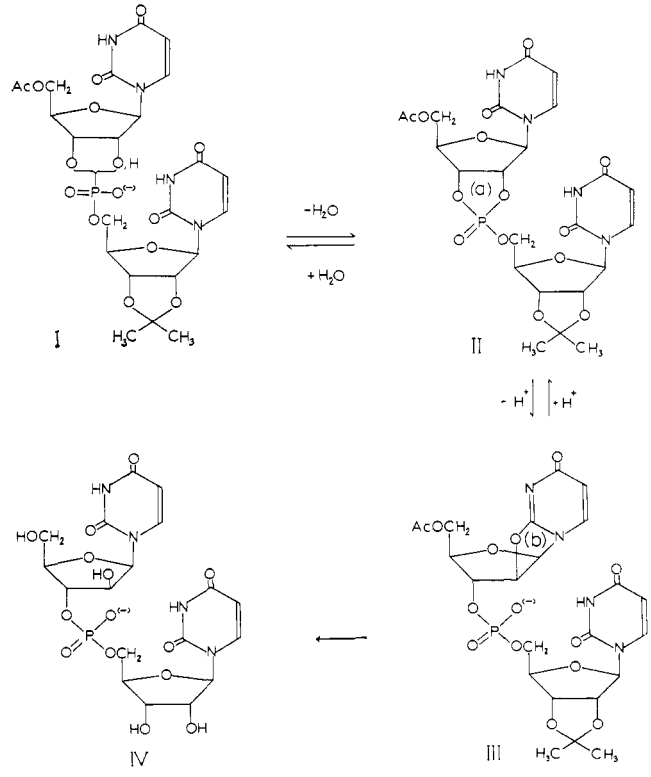
SCHEME II



to Michelson (1959). Because of its instability in aqueous solutions, the cold acetylation mixture was concentrated and the 5′-*O*,N^3-diacetyluridine 2′:3′-cyclic phosphate was precipitated with ether, washed with ether, and then used directly for synthesis. 5′-*O*-acetyluridine 2′:3′-cyclic phosphate was obtained from the diacetyl derivative in quantitative yield when it was chromatographed on silica gel as described below.

Preparation of 2′:3′-O-Isopropylideneuridine 5′-Phosphate. Uridine 5′-phosphate, disodium salt from the Sigma Chemical Co., was converted into bistri-*n*-butylammonium salt, which was dried by evaporation from dimethylformamide. After the addition of 2 equiv of *p*-toluenesulfonic acid and a large excess of acetone, the mixture was stirred for 2 hr at room temperature. The *isopropylideneuridine 5′-phosphate*, obtained after precipitation with tri-*n*-butylamine and ether, appeared as a single spot on paper chromatograms, and was used directly for further synthesis.

5′-O-Acetyluridine was prepared by the method of Brown *et al.* (1956), and *1-β-*D*-arabinofuranosyluracil* was purchased from Calbiochem.

Diphenyl phosphorochloridate, methanesulfonyl chloride, and *p*-toluenesulfonyl chloride were purified by distillation or recrystallization. Chloride-free tetraphenyl pyrophosphate was prepared by partial hydrolysis of diphenyl phosphorochloridate and fractional distillation (200–210°, 0.1 mm), or by the reaction of diphenyl phosphorochloridate with 1 equiv of silver diphenyl phosphate in toluene under shaking for 12 hr at 20°. In the latter case, the stock solution was filtered and used directly. Analytical grade dioxane, *N*,*N*-dimethylformamide, and tri-*n*-butylamine (Bu_3N) were first shaken with P_2O_5, then with KOH, and, after filtration through molecular sieves (Linde, Type 4A), dioxane was distilled at normal pressure, while the latter were distilled twice *in vacuo*. *N*,*O*-Bis(trimethylsilyl)acetamide was a product of Pierce Chemical Co.

Methods. Descending paper chromatography was carried out on Whatman No. 1 paper in the following solvent mixtures: 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–ethanol–0.5 M ammonium acetate (pH 7.2) (5:3:2) (D).

Thin-layer chromatography was performed on Eastman Chromagram sheets with fluorescent indicator in the solvent mixtures 1-butanol–acetone–water (4:2:1) and 1-butanol–acetone–1 M aqueous triethylammonium bicarbonate (pH 8) (2:1:1).

Electrophoresis of nucleotides was carried out in a Savant flat-plate apparatus at 2000 V in 0.05 M phosphate buffer (pH 7.3). The electrophoretic separation of nucleosides was performed according to Gordon *et al.* (1958).

Separation of the synthetic products on a preparative scale (1 mmole) was done by ion-exchange chromatography in the earlier phase of this work, and followed the method of Taylor and Hall (1964). Later silica gel chromatography was more successfully employed for the isolation of pH-sensitive cyclouridine derivatives. The silica gel (80–200 mesh) was washed consecutively with 1 M triethylammonium bicarbonate (pH 9), water, and acetone, and air dried. The column (75 × 2.5 cm) was packed with silica gel in water-saturated butanol; the reaction mixture was applied to it in 1-butanol–acetone–water (4:2:1) and eluted with a 4:4:1 mixture of the same solvents below 0°. The fractions were concentrated under vacuum below 0°, and the nucleotides were precipitated with ether. This procedure is particularly suitable for the isolation of labile derivatives of mono- and dinucleotides.

Nuclear magnetic resonance spectra were taken on a Varian HA 100 spectrometer in D_2O, using tetramethylsilane as an external standard. Ultraviolet spectra were taken in water using a Beckman DU spectrophotometer. The optical rotations were measured on a Cary 60 spectropolarimeter.

Enzymatic Hydrolyses. Purified venom phosphodiesterase, spleen phosphodiesterase, and bacterial alkaline phosphatase were the products of Worthington Biochemicals Co. Ten optical density units of substrate in 0.1 ml of 0.1 M Tris buffer (pH 9.0) for the venom diesterase and phosphatase assays and 0.2 M ammonium acetate (pH 5.8) for spleen diesterase were incubated with an excess of enzymes at 37° for 5 hr. The protein was precipitated by boiling or with chloroform prior to separation by the techniques described above.

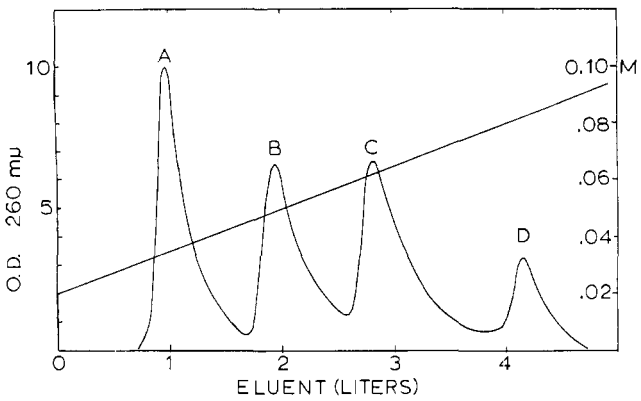


FIGURE 1: Separation of a mixture containing an arbitrary ratio of cU-rU (III, peak A), the isomeric rU-rU (II, peaks B and C), and aU-rU (IV, peak D), totaling approximately 1 mmole, on Dowex 1-X2 column (42 × 1.2 cm., formate) with a linear gradient of ammonium formate pH 4.5 according to Taylor and Hall (1964).

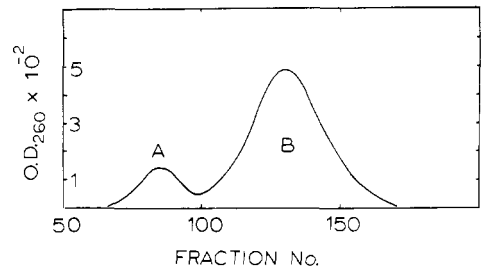


FIGURE 2: Separation of rU-rU (I, peak A) and cU-rU (III, peak B) on silica gel column (60–200 mesh, 75 × 2.5 cm) in a mixture of 1-butanol–acetone–water (4:4:1, v/v); 5-ml fractions were collected; total amount 0.4 mmole.

Drying Procedure. The reaction mixtures were rendered anhydrous by three evaporations from dimethylformamide *in vacuo*. The gummy residues were dissolved in the solvent of choice, Bu_3N was added, and the solutions were transferred in bulbs which had been freshly dehydrated by heating *in vacuo* at 200° and sealed after the addition of an activating agent. Critical manipulations were carried out in a glove box.

Synthetic Approaches. All syntheses of arauridylyl-(3′→5′)-uridine (aU-rU,[1] IV, Scheme II) to be described here are based upon the intermediate formation of the cyclic triester, 5′-*O*-acetyluridylyl-[(2′:3′)→5′]-(2′:3′-*O*-isopropylidene)-uridine (III).

(1) The original method of Michelson (1963) for the preparation of oligonucleotides *via* cyclic triester has been applied with some modifications. The starting materials, 1 mmole of tri-*n*-butylammonium 5′-*O*-acetyluridine 2′:3′-cyclic phosphate and 1.3 mmoles of 2′:3′-*O*-isopropylideneuridine, were dissolved in a mixture of 4 ml of dioxane, 4 ml of dimethylformamide, and 1.2 ml of Bu_3N. Diphenyl phosphorochloridate or tetraphenyl pyrophosphate, 1.5 mmoles, was added to the solution under cooling, which was then kept at room temperature overnight, and subsequently heated at 90° for 10 hr. The best yield of cU-rU (III), around 90%, can generally be obtained by slight variations in the amount of the activating agent.

(2) In the second *de novo* synthesis a small excess of 5′-*O*-acetyluridine was reacted with 2′:3′-*O*-isopropylideneuridine 5′-phosphate in dimethylformamide–Bu_3N mixture under the slow addition of 3 equiv of *p*-toluenesulfonyl chloride over a period of 2 days at room temperature. The usual thermal conversion (90°, 10 hr) yielded only 20% and 17% cU-rU in two attempts.

(3) Conversion of rU-rU into aU-rU (Scheme II, I → II → III → IV). 5′-*O*-Acetyluridylyl-[2′(3′)→5′]-(2′:3′-*O*-isopropylidene)uridine (I) which was obtained according to Taylor and Hall (1964) was dissolved in dioxane or dioxane–dimethylformamide mixtures, and activated with a variety of agents, such as diphenyl phosphorochloridate, tetraphenyl pyrophosphate, *p*-toluenesulfonyl chloride, and methanesulfonyl chloride in the presence of tri-*n*-butylamine. Conditions and yields are discussed under Results.

Since the nucleoside 2′:3′-cyclic triesters are extremely susceptible to hydrolysis (Michelson, 1959), no attempt was made to isolate triester II from the anhydrous solutions. The conversion of triester II into cU-rU (III) was studied as a function of temperature, time, solvent, and concentration of activating agents. In the kinetic studies, usually carried out at 90°, two duplicates were always prepared. One of them was worked up at varying times, while the other tube served to measure the maximum possible conversion in that particular mixture, generally after 8–10 hr. The eventual excess of activating agent was hydrolyzed in the cold with pyridine–water, and the solution was evaporated to a gum which was repeatedly extracted with ether prior to chromatography.

Pure cU-rU can only be obtained through careful operation. Although it is well separated by ion-exchange chromatography on Dowex 1-X2 (formate form) (Figure 1), a partial conversion into rU-rU takes place on freeze drying. Chromatography on silica gel in a saltless solvent mixture is the mildest isolation procedure and allows good separation (Figure 2), provided that no 5′-*O*-acetyluridine 2′:3′-cyclic phosphate is present. If this starting material has not reacted completely, a pancreatic ribonuclease treatment is necessary which will hydrolyze it to the separable monoester.

Alkaline hydrolysis of cU-rU was carried out in an excess of 1 N KOH at 20° for 20 min, then the solution was adjusted to pH 1 with perchloric acid for further deblocking, which was chromatographically followed. The extent of conversion II → III was expressed, (a) as the ratio of the amounts of aU-rU isolated by paper chromatography following hydrolysis from the reactions at the given time and after 10 hr, and (b) as the ratio of the cU-rU content of the duplicates measured from the ion-exchange and silica gel chromatographic patterns (see Figures 1 and 2).

Results

Characterization of the Reaction Products. The two new compounds obtained by all three approaches were identified

[1] The following abbreviations were employed in accordance with the Extended Rules of the IUPAC–IUB Commission on Biochemical Nomenclature (Sober, 1968) as suggested to us by Dr. W. E. Cohn: rU, 1-β-D-ribofuranosyluracil, uridine; aU, 1-β-D-arabinofuranosyluracil, arauridine; cU, O^2:2′-cyclouridine. The dinucleoside monophosphates were designated without reference to protecting groups as follows: rU-rU, 5′-*O*-acetyluridylyl-[2′(3′)→5′]-(2′:3′-*O*-isopropylidene)uridine; aU-rU, arauridylyl-(3′→5′)-uridine; cU-rU, 5′-*O*-acetyl-O^2:2′-cyclouridylyl-(3′→5′)-(2′:3′-*O*-isopropylidene)uridine.

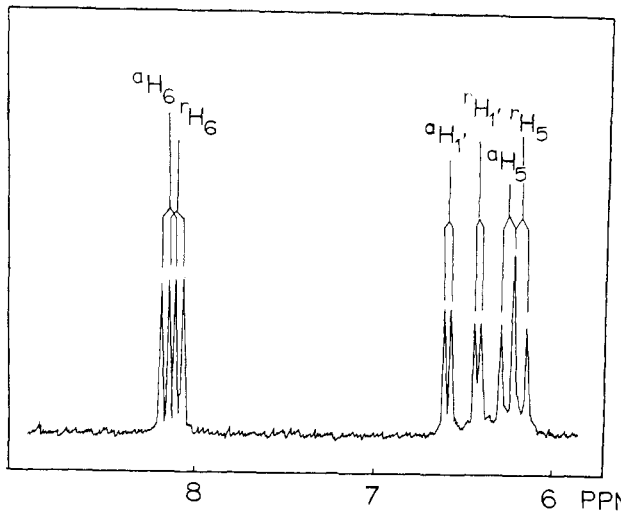


FIGURE 3: Characteristic nuclear magnetic resonance signals of aU-rU (IV). The 100-Mcps spectrum was taken on a Varian HA 100 spectrometer at a sweep width of 1000 cps in D_2O, using tetramethylsilane as an external standard. The abbreviations ^aH_i and ^rH_i stand for the corresponding protons in the aU and rU moieties. The numerical values of δ and J are given in Results.

as 5′-*O*-acetyl-O^2:2′-cyclouridylyl-3′→5′)-(2′:3′-*O*-isopropylidene)uridine (cU-rU, III) and arauridylyl-(3′→5′)-uridine (aU-rU, IV). The hydrolysis of the cyclic isourea ether linkage in cU-rU by a great excess of 0.2 N KOH at room temperature is complete in 20 min. Subsequent acidic deblocking yielded aU-rU in virtually quantitative yield, which was isolated as the barium salt. The analysis of the free acid indicated only a small amount of impurities. *Anal.* Calcd for $C_{18}H_{23}N_4O_{14}P \cdot 1.5H_2O$ (577.39): C, 37.45; H, 4.54; N, 9.70. Found: C, 37.91; H, 4.83, N, 9.06. The molar rotation, $[M]_D^{25}$ 330° ($c = 8.96 \times 10^{-5}$ M) is in good agreement with the sum of the individual residue rotations (310° + 23° = 333°).

Both compounds were entirely hydrolyzed by snake venom phosphodiesterase. The hydrolysis of cU-rU produced 5′-*O*-acetyl-O^2:2′-cyclouridine which was recognized by its broad ultraviolet maximum at 250 mμ. The 5′-nucleoside from aU-rU was crystallized from methanol and identified as 1-β-D-arabinofuranosyluracil by the following criteria: the optical rotation $[\alpha]_D^{20}$ +128° (*c* 1, in water, before crystallization; lit. (Brown *et al.*, 1956) $[\alpha]_D^{20}$ +131.1°), the melting point, 222–225° (Calbiochem sample 223–226°), the paper chromatographic behavior, and the identical mobility in electrophoresis with an authentic sample in the borate system of Gordon *et al.* (1958) which allows the differentiation of all four isomers. It is interesting to note that, while aU-rU was slowly but completely degraded by spleen diesterase, cU-rU remained intact. Neither aU-rU nor cU-rU was hydrolyzed by pancreatic RNase.

The 100-Mcps nuclear magnetic resonance spectrum (Figure 3) of aU-rU is in full agreement with the proposed structure. It shows both the characteristic downfield shift of the C-1′ proton on the arabino moiety (Broom *et al.*, 1967) and a complete resolution of the C-5 and C-6 protons of the rU and aU. The signals at δ 6.58 (J = 4 cps), 6.25 (J = 8 cps), and 8.14 ppm (J = 8 cps) have been attributed to the H-1′, H-5, and H-6 in aU, while the corresponding signals in rU appear at δ 6.42 (4), 6.18 (8), and 8.10 ppm (8).

Our nuclear magnetic resonance spectrum of cU-rU is similarly conclusive, although less accurate. During the extensive purification a part of the protecting groups has been lost, and this could have caused the broadening of some of the signals. This spectrum relates to acetone as an internal standard and contains doublets at δ 5.69 (8), 5.56 (8), and 3.99 (8) ppm; a poorly resolved peak, possibly a doublet, at 4.33; a multiplet of two doublets both centered at 3.66 (J = 4–5 and 8 cps, respectively); a triplet at 3.42 (5 cps). The assignment of these signals was facilitated by the use of double resonance. Upon irradiation at 569 cps a singlet was obtained at 3.99, therefore the doublets at 5.69 and 3.99 ppm originate from the H-6 and H-5 of the same base. Similar relationship was established for the doublets at 5.56 and 3.66 ppm. We assign the first pair of doublets to the cyclouridine moiety which is expected to have the lower electron density and stronger deshielding effect from the two bases. For the same reason the broad signal at 4.33 ppm can be assigned to H-1′ of cU. Correspondingly, the doublets at 5.56 and 3.66 (J = 8, 8, and 4–5 cps) should originate from the H-6, H-5, and H-1′ of the rU moiety. The triplet at δ 3.42 can be tentatively assigned to H-2′ of cU, because this proton of the ribonucleotides always appear further upfield.[2]

Chromatographic, electrophoretic and ultraviolet data are listed in Table I.

The Comparison of Various Approaches. The three approaches discussed in this paper differ from one another only in the first step of synthesis which is the formation of the dinucleoside (2′:3′)→5′-cyclic triester (II).

From the *de novo* approaches, we found that our modification of the Michelson method (approach 1) is particularly facile. Michelson's method (Michelson, 1963) involves, in general, the activation of protected (or unprotected) nucleoside 2′:3′-cyclic phosphates, or oligonucleotidyl 2′:3′-cyclic phosphates in the presence of an unprotected 5′-nucleoside, or oligonucleotide; the hydrolysis of the intermediary cyclic triesters leads to the formation of mixed 2′(3′)→5′-oligonucleotides. Our modification consists mainly in heating the cyclic triester, in the present case 5′-*O*-acetyluridylyl-[(2′:3′)→5′]-(2′:3′-*O*-isopropylidene)uridine, for varying periods of time prior to hydrolysis. The first step, a condensation reaction, is virtually quantitative if the alcoholic component is in slight excess, and is complete within a few hours at room temperature. The yield of the second step, the thermal rearrangement, is 60–90%, which is quite satisfactory. Some factors affecting this rearrangement will be discussed further below.

Approach 2 is most difficult to handle, and the yields are the lowest. Since the reaction of the uridine 5′-phosphate with the first secondary hydroxyl group is relatively slow and some tosylation also takes place, the 3 equiv of *p*-toluenesulfonyl chloride must be added slowly. The lower yield could be due to difficulties in anticipating the proper amount of activating agent, because only the formation of the diester I, and not that of the triester, can be followed directly. This approach was explored in the hope to provide an alternative for the case if only the 5′-nucleotide is available or if the

[2] The very same deshielding of the H-2′ was found in the nuclear magnetic resonance spectra of oligo-cU and O^2:2′-cyclocytidine 3′-phosphate which were recently obtained in our laboratory.

TABLE I: Paper Chromatographic and Electrophoretic Mobilities.[a] Ultraviolet Data.

Compounds	R_P of Solvent Systems A	B	C	D	Electrophoresis[b]	λ_{max} (ϵ) in Water
rU-rU (II)			0.69	0.36	0.33	261 (19,200)
cU-rU (III)			0.69	0.36	0.32	255 (17,100)
aU-rU (IV)	0.11		0.51	0.11	0.38	262 (18,600)
Uridine 3′-phosphate	0.06		0.45		1.0	
2′:3′-*O*-isopropylidene-uridine 5′-phosphate	0.24			0.19		
5′-*O*-Acetyluridine 2′:3′-cyclic phosphate			0.65	0.32	0.65	258
Uridine	0.37	0.37				
1-β-D-Arabinosyluracil	0.45	0.45				262.5

[a] Conditions given in Procedures. [b] Relative to uridine 3′-phosphate.

incorporation of a nucleoside analog into the 5′ end position is desired, and in such cases it may be useful.

The conversion of rU-rU into aU-rU (approach 3) is a less expedient way of synthesis than the above *de novo* syntheses because the starting material has to be first prepared according to Taylor and Hall (1964). The merit of this idea (Scheme II, I → II → III → IV) is that it demonstrates in the clearest form the convertibility of the interribonucleotidic bond into an inter(arabin–oribo)nucleotidic bond. The application of this method to preformed oligo- and polynucleotides should enable a similar conversion of the existing linkages into a novel interarabinonucleotidic type. The usefulness of this model system in kinetic studies is described in the following section.

Kinetics of the Rearrangements II → III. In approaches 1 and 3 the triester II forms rapidly and quantitatively at room temperature, with only traces of contaminating cU-rU present. Hence, these systems are best suited to studying the thermal rearrangement from cyclic triester II to diester III. In spite of the considerable effort already invested in such studies, it has not been possible as yet to obtain reliable data on the kinetics and activation energy of the rearrangement. The reason for this is the large number of influencing factors, all of which are subject to variation with the individual technic and its improvement. The amount and nature of the activating agent, and the ratio of the remaining moisture to the employed excess of the activating agent is of particular importance. Within a certain set of conditions, the optimum yield for cU-rU is reached after 10 hr at 90°, or 16 hr at 75°. This optimum yield, however, is a function of the amount of the activating agent. Figure 4 shows that diphenyl phosphorochloridate can be added only in small excess, otherwise the yield of cU-rU rapidly diminishes. This phenomenon can be attributed in part to the nucleophilicity of the chloride ions. In the presence of 10 equiv of $Bu_3N \cdot HCl$, the internucleotide linkage is broken down, and only monomers can be detected. On the other hand, the rearrangement is almost equally poor with a great excess of tetraphenyl pyrophosphate, although the internucleotide linkage is more stable. A plausible explanation can be obtained by examining the reactivity of 5′-*O*,N^3-diacetyluridine 2′:3′-cyclic phosphate. From the lack of rearrangement in this case, one can generalize that the cyclic triesters of N^3-substituted nucleosides are stable at 90° for 8 hr. To further elaborate this point, the triester II was also heated in the presence of an excess of bis(trimethylsilyl)acetamide. As expected, the trimethylsilylated base was no longer nucleophilic, and no rearrangement was observed. In another experiment, the fully trimethylsilylated 5′-*O*,N^3-bis(trimethylsilyl)uridine 2′:3′-cyclic trimethylsilyl phosphate was also found stable up to 100°. The phosphorylation of the uracil moiety by the excess of the activating agent would similarly explain the diminished yields.

Since the reaction conditions are not easily reproduced, the real concentration of the N^3-unsubstituted cyclic triester varies in each experiment. It is plausible to assume, however, that the amount of cU-rU in one of the duplicates, which was opened after 10 hr, equals the reactive form of the cyclic triester originally available. Accordingly, a meaningful rate

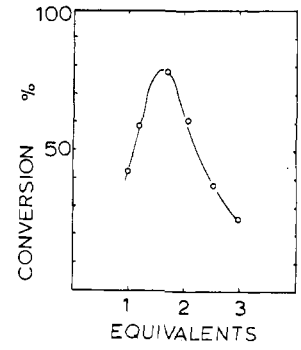


FIGURE 4: The conversion of the cyclic triester II into cU-rU (III) as a function of the amounts of activating agent used to its generation. The reaction mixtures consisted of 1 mmole of tri-*n*-butylammonium 5′-*O*-acetyluridine 2′:3′-cyclic phosphate, 1.3 mmoles of 2′:3′-*O*-isopropylideneuridine, 2 ml of Bu_3N, and the indicated amounts of diphenyl phosphorochloridate in 8 ml of dioxane. They were kept at 20° for 16 hr, then at 90° for 16 hr. The degree of rearrangement was determined by isolating the aU-rU (IV) after hydrolysis.

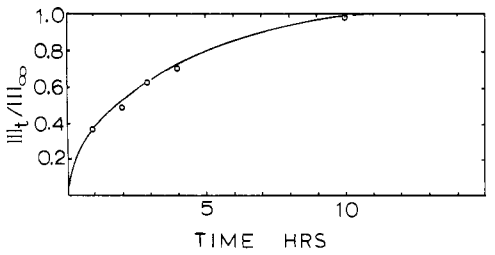


FIGURE 5: Progress curve for the rearrangement II → III, expressed as the ratio $III_t:III_\infty$ *vs.* time, where III_t and III_∞ indicate the amounts of cU-rU at a given time and after 10–16 hr, respectively. The reaction mixture consisted of 1 mmole of tri-*n*-butylammonium 5′-*O*-acetyluridine 2′:3′-cyclic phosphate, 1.3 mmoles of 2′:3′-*O*-isopropylideneuridine, 1.6 mmoles of diphenyl phosphorochloridate, 4 ml of dioxane, 4 ml of dimethylformamide, and 1.2 ml of Bu_3N. It was kept for 16 hr at 20° prior to heating at 90° for varying time.

curve could only be obtained when the amount of cU-rU at a given time $[III]_t$ was divided by the final and highest amount $[III]_\infty$ found in the same mixture. When using a mixture of dioxane and dimethylformamide as solvent under the conditions given for approach 1 in Figure 5, the half-time of the rearrangement is approximately 2 hr. Approach 3 gave similar results, but it exhibited an even greater sensitivity to the variations in the amounts of activating agent, because no excess of alcoholic groups were available here to bind the excess of activating agent. Since no monomers were present originally, approach 3 was most suitable for observing the side reaction causing degradation. The change of solvent from dioxane–dimethylformamide to pure dioxane increases the half-time of the rearrangement, and also causes a drop in the final yield. The change of the activating agent from diphenylphosphoryl to sulfonyl derivatives renders the rearrangement even more sensitive to excessive activation. It seems that the sulfonylation of the base is quite competitive with the esterification reaction due to the lesser steric requirement and the relative stability of the end product.

The Effect of Acid and Base on the Rearrangement II → III. Unpublished work from our laboratory had shown that the rate of the reaction of phospho mono- and diesters with O^2:5′-cyclouridine (Nagyvary, 1966) was enhanced by general acid catalysis. On the basis of this early observation it was felt that the cU-rU system would be unstable in the presence of anhydrous acid and should be reverted to, or be in equilibrium with II. Experimental difficulties due to the hydrolysis of the cyclonucleoside moiety in III preclude the exact measurement of such an equilibrium, but the reverse reaction III → II could be easily demonstrated.

When cU-rU was treated with 2 equiv of anhydrous *p*-toluenesulfonic acid in dimethylformamide at 60° for 1 hr and warmed after dilution with water at 37° overnight, 2′(3′)-uridylate and uridine were isolated in addition to 30% aU-rU by chromatography in solvent "A." The same hydrolysis products were isolated following 6-hr heating of III in trifluoroacetic acid in a ratio of 45:45:10. Since aU-rU is stable under these conditions, this finding can be explained only by assuming an inversion of the configuraton at C-2′, resulting in the sequence III → II → I. The hydrolysis of the phosphodiester linkage of cU-rU can be observed even in 1 N aqueous H_2SO_4 in trace amounts. In contrast to its behavior at high pH, the O^2:2′-cyclouridine moiety is more stable in 0.2 N HCl and 3 days are needed to its complete hydrolysis at room temperature.

Discussion

The convertibility of the ribo moiety into the arabino moiety is an inherent property of pyrimidine ribooligonucleotides, which raises a number of interesting theoretical and practical questions.

The driving force of the base-catalyzed thermal rearrangement II → III is, in part, the greater stabilization of the negative charge on the phosphodiester rather than on the base moiety. The completion of this reaction in the absence of acid is also due to the difference in ring strain between the rings a and b in the formulas II and III, respectively, favoring the O^2:2′-cyclonucleoside. The specificity of the attack by the C^2-0 at the C-2′, instead of the C-3′, may be in part due to a kinetic control, as is expected when assuming the *anti* conformation for the uracil moiety, but there are also some indications of a significant difference in the stabilities of O^2:2′ and O^2:3′-cyclonucleosides (Michelson and Todd, 1955).

One of the interesting aspects of this work is the discovery of the equilibrium II ⇄ III, which represents an acid–base-catalyzed reaction. The reverse reaction is due to the neighboring group participation of the phosphate anion, and its extent depends upon the water content. While increasing water concentration favors the formation of the arabino derivative, it is not advisable to hydrolyze the cyclonucleoside moiety in oligonucleotides with acid, as was done in the original procedure on O^2:2′-cyclouridine (Brown *et al.*, 1956). Our concept of equilibrium is not incompatible with the work of Walwick *et al.* (1959) and Schramm and Ulmer-Schurn brand (1967), in which the formation of cyclonucleoside derivatives was described in polyphosphoric acid and polyphenyl phosphate, both in the absence of base. The strongly polar nature of these solvents should promote the formation of the more polar endproduct. It is also likely that the cyclic pyrophosphate is a more reactive intermediate than the cyclic triester. The yield of the conversion in these two reactions was far from being quantitative, indicating an equilibrium mixture. It should be mentioned that Walwick (1958) also described the partial reversal of O^2:2′-cyclouridine 3′,5′-diphosphate into uridine 2′(3′),5′-diphosphate in hydrochloric acid.

The formation of aU-rU from cU-rU is an irreversible process, and no simple way to convert the arabinosyl derivative into the ribosyl derivative is apparent to us. On the other hand, it has not escaped our attention that a suitable inversion of the configuration on the C-2′ in cU-rU could lead to a new type of synthesis of the natural interribonucleotide bond. We are at present exploring this possibility, which may have some pertinence to the prebiological formation of the 3′→5′-linked polyribonucleotides.

The uniformly 3′→5′ nature of the phosphodiester linkages is a corollary of the synthesis, and was proved by the enzymatic degradations. The stability of the internucleotide linkage between arabinonucleosides was noted previously by Wechter (1967). The lack of hydrolysis by acid, alkali, and RNase is undoubtedly a consequence of the change of the configuration on C-2′. This change of configuration has

resulted in a qualitative change in the interaction of the $C^{2'}$-OH with the base. In the place of the weak hydrogen bond between C-2 oxygen and $C^{2'}$-OH in uridine, one can envisage a stronger complex between $C^{2'}$-OH and the π electrons of the base in arauridine. This assumption is supported by the observed downfield shift of the H_6 and H_5 protons of aU relative to those of rU in the 100-Mc proton magnetic resonance spectrum of aU-rU (Figure 3). The abnormality of the 3′→5′-interarabinonucleotidic linkage is further emphasized by the very sluggish hydrolysis in the presence of spleen phosphodiesterase. In this respect it is noteworthy that significant decrease was found in the rate of hydrolysis of the 5′-esters by an exonuclease from the venom of *Crotalus adamanteus* (Richards *et al.*, 1967).

The characterization of the 5′-nucleoside as 1-β-D-arabinofuranosyluracil is satisfactory, but the structure is not free of one uncertainty which surrounds the authentic material itself. Molecular models suggest that two stable rotational isomers, *syn* and *anti*, should exist. There is no doubt that the formation of the *anti* isomer is both kinetically and thermodynamically favored, even if not exclusively.

This study of the conversion of the ribo moiety into arabino moiety at the oligonucleotide level has been carried out on the simplest model, a dinucleoside monophosphate. Although our data on the exact kinetics of the conversion need further elaboration, most of the imponderables have been recognized and taken into consideration in the synthesis of polyarauridylic acid.

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References

Broom, A. D., Schweizer, M. P., and Ts'o, P. O. P. (1967), *J. Am. Chem. Soc. 89*, 3612.

Brown, D. M., Todd, A. R., and Varadarajan, S. (1956), *J. Chem. Soc.*, 2388.

Cohen, S. S. (1966), *in* Progress in Nucleic Acid Research and Molecular Biology, Vol. 5, Davidson, J. M., and Cohn, W. E., Ed., New York, N. Y., Academic, p 2.

Gordon, M. P., Interieri, O. M., and Brown, G. B. (1958), *J. Am. Chem. Soc. 80*, 5161.

Michelson, A. M. (1959), *J. Chem. Soc.*, 3655.

Michelson, A. M. (1963), The Chemistry of Nucleosides and Nucleotides, New York, N. Y., Academic, pp 419–440.

Michelson, A. M., Dondon, J., and Grunberg-Manago, M. (1962), *Biochim. Biophys. Acta 55*, 529.

Michelson, A. M., and Todd, A. R. (1955), *J. Chem. Soc.*, 816.

Nagyvary, J. (1966), *Biochemistry 5*, 1316.

Nagyvary, J. (1967a), *Fed. Proc. 26*, 292.

Nagyvary, J. (1967b), XXI Congress of IUPAC, Prague, Abstracts N-48.

Nagyvary, J. (1968), Symposium on Nucleic Acids and Proteins, Houston, Texas, University of Houston.

Nagyvary, J., Provenzale, R., and Clark, J. M. (1968), *Biochem. Biophys. Res. Commun. 31*, 508.

Renis, H. E., Hollowell, C. A., and Underwood, G. E. (1967), *J. Med. Chem. 10*, 777.

Richards, G. M., Tutas, D. J., Wechter, W. J., and Laskowski, Sr., M. (1967), *Biochemistry 6*, 2908.

Schramm, G., and Ulmer-Schurnbrand, I. (1967), *Biochim. Biophys. Acta 145*, 7.

Sober, H. A., Ed. (1968), *in* Handbook of Biochemistry, Cleveland, Ohio, Chemical Rubber, pp A8–9, 63–8, H14–19, and H62–5.

Taylor, P. R., and Hall, R. H. (1964), *J. Org. Chem. 29*, 1078.

Walwick, E. R. (1958), Ph.D. Thesis, University of California, Berkeley, Calif.

Walwick, E. R., Roberts, W. K., and Dekker, C. A. (1959), *Proc. Chem. Soc.*, 84.

Wechter, W. J. (1967), *J. Med. Chem. 10*, 762.