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THE SYNTHESIS OF OLIGORIBONUCLEOTIDES—IV*

PREPARATION OF DINUCLEOSIDE PHOSPHATES FROM 2',5'-PROTECTED RIBONUCLEOSIDE DERIVATIVES

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Abstract—The preparation of 2'-O-tetrahydropyranyl-uridine and -adenosine is described. Both diastereoisomers of each derivative have been isolated in a pure crystalline state. The high-melting (more laevorotatory) diastereoisomer derived from each nucleoside has been converted to its 5'-O-pivaloyl derivative. Both diastereoisomers of 2'-O-tetrahydropyranyl-5'-O-pivaloyluridine have been independently prepared from 3'-O-acetyl-5'-O-pivaloyluridine, condensed with 2',3'-O-methoxymethylideneuridine 5'-phosphate, and thereby converted into uridylyl-(3' → 5')-uridine in good yield. The 2'-O-tetrahydropyranyl protecting group may be removed by acidic hydrolysis (with 0·01N HCl) under very mild conditions which lead to a negligible amount of isomerization (by phosphoryl migration) and degradation of the dinucleoside phosphate.

Each of the dinucleoside phosphates: adenylyl-(3' → 5')-uridine, uridylyl-(3' → 5')-adenosine, uridylyl-(3' → 5')-cytidine and adenylyl-(3' → 5')-cytidine has also been prepared in the same way from the appropriate 2'-O-tetrahydropyranyl-5'-O-pivaloyl nucleoside.

FOR THE past few years, one of the most active areas of research in nucleic acid chemistry has been that concerned with the development of suitable procedures for the synthesis of short oligoribonucleotides of known base-sequence. The importance of such investigations has recently been emphasized by the discovery[1] that tri-ribonucleoside diphosphates may be used in the elucidation of the genetic code.

The crucial problem of oligoribonucleotide synthesis is that all the 2'-OH functions must be protected until the final step, and in such a manner that they may then be released under very mild conditions. Only in this way can it be ensured that the nucleotide units are joined together exclusively by 3' → 5'-phosphodiester linkages. This has proved to be a complex problem and, despite the availability of adequate condensation methods,[2] progress has been relatively slow. However, several successful approaches have now been reported[3–5] and it is our purpose in the present paper to discuss one of them in detail, and more particularly to describe its application to the synthesis of di-ribonucleoside phosphates.

The two most obvious routes for the synthesis of di-ribonucleoside phosphates are illustrated in chart 1: route (a), which has been used by all the other workers†

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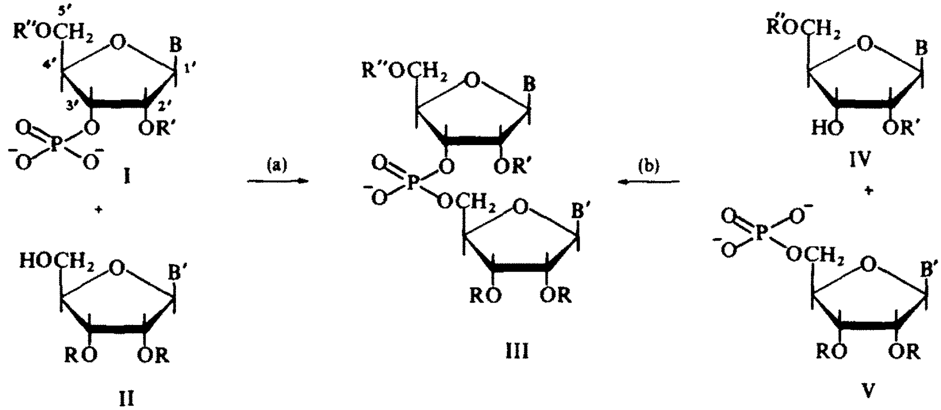
* For part III of this series, see H. P. M. Fromageot, B. E. Griffin, C. B. Reese, and J. E. Sulston, *Tetrahedron*, 23, 2315 (1967).

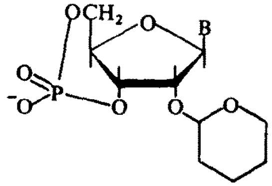
† Ukita *et al.*[6] have recently described a route (b) condensation between 2',5'-di-O-trityluridine and 2',3'-di-O-acetyluridine 5'-phosphate, but have demonstrated that it is not possible to remove the trityl protecting groups without causing considerable migration of the 3' → 5' internucleotide linkages.

in this field,[3,4,7-9] involves the condensation between a 2′,3′-protected ribonucleoside (II) and a 2′,5′-protected ribonucleoside 3′-phosphate (I), and route (b) which involves the condensation between a 2′,5′-protected ribonucleoside (IV) and a 2′,3′-protected ribonucleoside 5′-phosphate (V). We have concentrated exclusively on the latter approach.

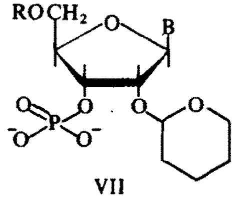
Chart 1



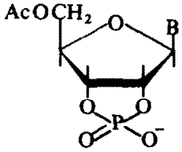
The two principal factors which determined the initial choice between routes (a) and (b) were the availability of suitably orientated nucleoside and nucleotide intermediates, and the nature of the protecting groups required. It is not, as yet, possible to gauge the importance of a third factor: i.e. the relative advantages of phosphorylating a 5′-OH group with an activated 3′-phosphate or of phosphorylating a 3′-OH function with an activated 5′-phosphate.



VI



VII



VIII

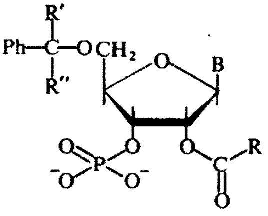
At the outset there was no known 2′,5′-protected intermediate (I or IV) which could be used directly in either a route (a) or a route (b) synthesis. However, the potential value of ribonucleoside 3′,5′- and 2′,3′-cyclic phosphates as sources of the 2′,5′-protected 3′-nucleotide components (I) in a route (a) synthesis was realized in two laboratories.[10,11] Khorana *et al.*[10] prepared 2′-O-tetrahydropyranyl derivatives of ribonucleoside 3′,5′-cyclic phosphates (VI) and showed that they underwent hydrolysis in hot aqueous baryta solution to give mainly the corresponding 3′-phosphates (VII; R = H). The latter were then protected on the 5′-position to give the required intermediates VII. Smrt and Šorm[11] prepared 5′-O-acetyl derivatives*

* In the case of cytidine 2′,3′-cyclic phosphate, the N^4, $O^{5'}$-diacetyl derivative was obtained.

of uridine and cytidine 2′,3′-cyclic phosphates (VIII), which underwent quantitative hydrolysis to the corresponding 3′-monophosphates in the presence of pancreatic ribonuclease. The required components (VII; B = uracil or N^4-acetylcytosine, R = Ac) were prepared by the acid-catalyzed reaction between the latter 3′-nucleotide derivatives and 2,3-dihydro-4*H*-pyran.

The work of Smrt, Šorm and their co-workers in this field[4] has centred around the preparation of intermediates of the type VII (R = Ac) and their use in a route (a) synthesis. If the *cis*-diol system of the terminal nucleoside residue (II) is blocked by a base-stable (acid-labile) protecting group, then treatment of the initial condensation product (III; R″ = Ac) with base will free only the 5′-OH group of the other residue to give III (R″ = H). The latter protected dinucleoside phosphate may then be condensed with another 2′,5′-protected 3′-nucleotide unit (VII: R = Ac), and so the oligonucleotide chain may be extended in a stepwise manner.[4]

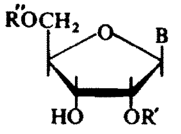
Khorana *et al.*, soon abandoned their original route (a) synthesis[10] which involved a 2′-O-tetrahydropyranyl-5′-protected-3′-nucleotide (VII) as the key intermediate. Although this approach could conceivably have been modified and thus made suitable for a stepwise synthesis, these workers concluded[12] that the acidic conditions necessary for the removal of the tetrahydropyranyl group were drastic enough to lead to appreciable migration of the 3′ → 5′-internucleotidic linkages (see below). For this reason they preferred 2′-O-acyl protecting groups[13] which were removable by base-catalyzed hydrolysis under very mild conditions. Thus, in nearly all of their subsequent work in this field,[3] Khorana *et al.* have followed a stepwise route (a) synthesis in which the key intermediates have been 2′-O-acyl-5′-O-triarylmethyl-3′-nucleotides[14] (IX; R = Me or Ph, R′ = p-MeO—C_6H_4, R″ = Ph or p-MeO—C_6H_4). These protected nucleotides have been condensed with the appropriate 2′,3′-di-O-acyl nucleoside derivatives (II; R = Ac or Bz) to give the fully protected dinucleoside phosphates (III). Treatment of the latter with aqueous acetic acid gave protected intermediates with free terminal 5′-OH groups (III; R″ = H) which could then be further condensed with either 3′-nucleotide units (IX) or with 2′,5′-di-O-acyl nucleoside 3′-phosphates.[13]

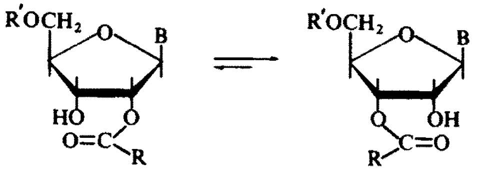


IX

Cramer *et al.*[7] have adopted the approach of Smrt and Šorm[11] with certain modifications. However, most of the products obtained[15] were found to be contaminated with appreciable quantities of the undesired isomers, containing 2′ → 5′-internucleotidic linkages. Like Khorana *et al.*, these workers have attributed their results to the severity of the acidic conditions required for the removal of the tetrahydropyranyl group; they have suggested[15] that the latter would be an unsuitable protecting group in an oligoribonucleotide synthesis designed to go beyond the dinucleoside phosphate stage. In their most recent work, Smrt and Šorm *et al.*[16] have also rejected the tetrahydropyranyl group in favour of a slightly more labile acyclic acetal system.

From the outset,[5] we have considered route (b) (chart 1) to be the more fundamental approach to oligoribonucleotide synthesis inasmuch as it involves the study of the selective reaction and protection of the *ribonucleoside* system itself. The development of methods for distinguishing between the secondary OH functions of the *cis*-2′,3′-diol system is, in addition to its relevance to oligoribonucleotide synthesis, clearly an important aspect of ribonucleoside chemistry. We therefore set out to prepare a series of pure crystalline 2′-protected ribonucleoside derivatives (IV; R″ = H), and to convert them into suitable components (IV; R″ ≠ H) for the initial step of a route (b) synthesis.


IV


X XI

The choice of the 2′-blocking group is crucial and determines the nature of all the other protecting groups to be used. There are at least three obvious possibilities to be considered: base-labile (e.g. acyl), hydrogenolyzable (e.g. benzyl ether), and acid-labile (e.g. acetal and ketal) blocking groups. Even if the necessary intermediates had been available, 2′-O-acyl protecting groups would initially have been considered to be unsuitable on the grounds that acyl migration was known to occur readily in 1,2-diol systems.[17] Furthermore, the earlier literature suggested[18] that the proportion of 3′-isomer (XI) considerably exceeded that of 2′-isomer (X) in an equilibrium mixture of 2′ (and 3′)-O-acyl ribonucleoside derivatives. For the latter reason and because of the apparent isomerization of $N^4,O^{2'},O^{5'}$-tribenzoylcytidine during phosphorylation, Rammler and Khorana[12] rejected the possibility of using a 2′-O-acyl protecting group in a route (b) synthesis. However, it has recently been shown[19] that, in several instances, 3′-acetates and benzoates (XI; R = Me and Ph, respectively) predominate only marginally over their corresponding 2′-isomers (X) at equilibrium, and thus that the latter are only slightly less stable under conditions which promote acyl migration. It is further apparent from equilibration rate studies[19] that the benzoyl group migrates slowly enough under phosphorylation conditions to make it suitable for the protection of the 2′-OH function in a route (b) synthesis.* However, it seems unlikely that the general use of the 2′-O-acyl protecting

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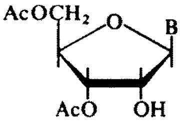
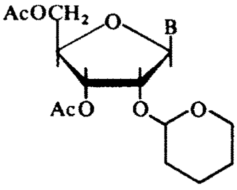
* This has since been confirmed[20] by the preparation of isomerically pure guanylyl-(3′ → 5′)-uridine from $N,O^{2'},O^{5'}$-tribenzoylguanosine[20] and 2′,3′-di-O-acetyluridine 5′-phosphate with mesitylenesulphonyl chloride[21] as the condensing agent, and with a reaction time of 6 hr in the phosphorylation step. Even if the shorter phosphorylation time is taken into account, this result is not in accord with the conclusions of Rammler and Khorana,[12] following their studies with $N^4,O^{2'},O^{5'}$-tribenzoylcytidine. However, we have so far[22] succeeded in isolating only pure $N^4,O^{3'},O^{5'}$-isomer from a mixture of $N^4,O^{2'}$ (and $O^{3'}$) $O^{5'}$-tribenzoylcytidines, and have noted that the two isomers tend to co-crystallize. Thus the possibility that the above $N^4,O^{2'},O^{5'}$-tribenzoylcytidine[12] was contaminated with $N^4,O^{3'},O^{5'}$-isomer cannot be discounted.

group is, at present, feasible in this latter approach. In the first place it requires that special precautions be taken to minimize acyl migration during phosphorylation, and secondly, it requires the availability of certain ribonucleoside derivatives which are not readily accessible by known synthetic procedures.

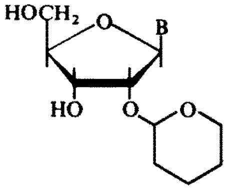
We recently prepared[23] uridylyl-(3′ → 5′)-uridine from a derivative of 2′-O-benzyluridine by a route (b) synthesis. Although in this particular case, the benzyl ether grouping in the protected dinucleoside phosphate was cleaved by catalytic hydrogenolysis, it cannot be assumed that de-benzylation would always be so easy to effect. Other factors which suggest that the benzyl ether might not be a generally suitable 2′-protecting group in a route (b) synthesis are, first, the susceptibility of the 5,6-double bond of cytosine and uracil residues to catalytic hydrogenation,[24] and secondly, the difficulty presently envisaged in preparing all the necessary 2′-O-benzyl-ribonucleoside derivatives, especially those of guanosine and adenosine.



XII



XIII



XIV

a; B = uracil-1
b; B = adenine-9

It therefore seemed that an acid-labile acetal or ketal group would be the best general choice for the protection of the 2′-OH function and, even if the suggested[12, 15, 16] unsatisfactory lability of the tetrahydropyranyl group were confirmed, it seemed likely that alternative more acid-labile systems could be found. Initially, the preparations of 2′-O-tetrahydropyranyl-uridine and -adenosine (XIVa and XIVb, respectively) were undertaken. The required starting materials, 3′-5′-di-O-acetyl nucleosides[18] (XIIa and XIIb), were readily prepared by the orthoester exchange procedure;[22, 25] their orientations were confirmed by NMR spectroscopy.[26]

3′-5′-Di-O-acetyluridine (XIIa) was found to react rapidly and quantitatively with 2,3-dihydro-4*H*-pyran in the presence of a small quantity (ca. 0·1 mole equiv with respect to nucleoside derivative) of toluene-*p*-sulphonic acid in dioxan solution to give XIIIa, which was then treated with methanolic ammonia. A solution of the ammonolysis products, in absolute ethanol deposited a crystalline compound with m.p. 185–187°. However, examination of the crude products by TLC revealed the presence of approximately equal quantities of two components, the less mobile of which corresponded to the above crystalline compound. This mixture was readily separated into its two constituents by chromatography on silicic acid: the constituent eluted first (corresponding to the more mobile component on TLC) crystallized from ethyl acetate as colourless needles, m.p. 146–148°. Both compounds had the elemental composition calculated for tetrahydropyranyluridine and, since 3′,5′-di-O-acetyluridine (XIIa) was not expected to isomerize under the experimental conditions, it was concluded that they were diastereoisomers of 2′-O-tetrahydropyranyluridine (XIVa). This conclusion has been confirmed by converting a 5'-protected derivative of each compound into a 3' → 5'-dinucleoside phosphate (see below). A completely stereospecific reaction between (XIIa) and 2,3-dihydro-4*H*-pyran would have been unexpected and, in any case, diastereoisomeric mixtures of tetrahydropyranyl derivatives of steroids[27] and sugars[28] have been previously been obtained.

In the same way, 3',5'-di-O-acetyladenosine (XIIb) was converted into a mixture of diastereoisomers of 2'-O-tetrahydropyranyladenosine (XIVb). In the first stage of this preparation, a slight excess (with respect to nucleoside derivative) of toluene-*p*-sulphonic acid was required but, in other respects, the procedure was similar to that adopted for the uridine derivative. The two diastereoisomers of 2'-O-tetrahydropyranyladenosine were separated by silicic acid chromatography and had m.p's 171–172·5° and 199–201° (Table 1); the latter compound had the lower mobility on TLC.

TABLE 1. PROPERTIES OF DIASTEREOISOMERS OF 2'-O-TETRAHYDROPYRANYL-RIBONUCLEOSIDES

Nucleoside	M.p.	$[\alpha]_D^a$	H(1')[b]	λ_{max}	(log ε)[c]
Uridine (i)	146–148°	+106°	4·10	262	(4·09)
Uridine (ii)	185–187°	− 47°	3·95	262	(3·98)
Adenosine (i)	171–172·5°	− 8°	3·89	259	(4·05)
Adenosine (ii)	199–201°	−130°	3·86	258	(4·16)

[a] Specific rotations were measured at 22–23° in aqueous (for uridine derivatives) or 95% ethanol (for adenosine derivatives) solution, at convenient concentrations (see experimental section).

[b] Chemical shifts are given in ppm on a τ scale. NMR spectra were run on dimethylcyanamide -D_2O (9:1; v/v) and dioxane-D_2O (3·1; v/v) solutions for uridine and adenosine derivatives, respectively.

[c] UV absorption spectra of aqueous solutions are given. The wavelengths of maximum absorption are in mμ.

Distinctions other than of m.p., TLC mobilities and solubility properties can be made between pairs of diastereoisomers. From Table 1, it can be seen that specific rotations, chemical shifts of H(1') resonances and intensities of the UV absorption maxima of each pair (i and ii) differ. The most striking feature of these data is that the high-melting diastereoisomers (ii) are considerably more laevorotatory than the low-melting diastereoisomers (i). It is further noteworthy that the specific rotations of the parent nucleosides* lie between those of the low- and high-melting 2'-O-tetrahydropyranyl derivatives. It would therefore seem likely that the absolute configuration of the tetrahydropyranyl asymmetric carbon centre is the same for the two low-melting and for the two high-melting diastereosiomers. We had previously observed[26] that the introduction of a 2'-substituent (or protecting group) into a ribonucleoside normally has a measurable deshielding effect on the glycosidic proton [H(1')]: this effect appears to be greater for the high-melting 2'-O-tetrahydropyranyl derivatives (ii).

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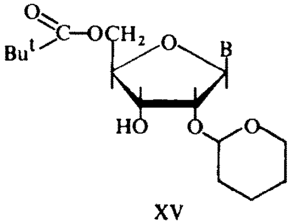
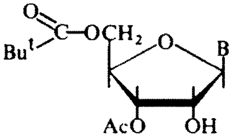
* Uridine has $[\alpha]_D^{16} = +9{\cdot}6°$ (c, 2 in H_2O);[29] adenosine has $[\alpha]_D^{20} = -68{\cdot}5°$ (c, 2 in 5% NaOH).[29]

As it is intended that the synthetic oligoribonucleotides should contain only 3′ → 5′-phosphodiester linkages, it is a necessary precaution to use only pure crystalline 2′-protected ribonucleoside derivatives as intermediates. It is therefore disadvantageous that two diastereoisomeric 2′-O-tetrahydropyranyl derivatives should be obtained, as this then necessitates the separation of a mixture and, more seriously, makes it unlikely that the isolated yield of either pure crystalline compound should exceed 50%. Thus, in principle, it would be more satisfactory to use a symmetrical 2′-protecting group. However, it has been found that the high-melting 2′-O-tetrahydropyranyl derivatives may generally be isolated from a mixture with little difficulty. For this reason, the latter compounds have been used in most of the synthetic work described below.

If an acid-labile 2′-protecting group is used in a route (b) synthesis (Chart 1), and it is intended to extend the dinucleoside phosphate from the terminal 5′-position, it is necessary that the nucleotide derivative (V) should also have an acid-labile 2′,3′-protecting group (or groups), and that the nucleoside derivative (IV) should have a 5′-residue which is base-labile. As acylation of a 2′-O-tetrahydropyranylnucleoside (XIV) with a stoicheiometric quantity of acetic anhydride would be expected* to give an appreciable amount of its 3′,5′-diacetate (XIII) in addition to a mixture of 5′- and 3′-monoacetates, it seemed reasonable to use a bulkier acyl group.



XV



XVI

a; B = uracil-1
b; B = adenine-9

When the high-melting diastereoisomer of 2′-O-tetrahydropyranyluridine (XIVa) was treated with a small excess of pivaloyl chloride in pyridine solution, and the products fractionated by silicic acid chromatography, 2′-O-tetrahydropyranyl-5′-O-pivaloyluridine (XVa) was obtained as a pure crystalline solid (m.p. 174–176°) in 47% yield. It was later found that the products of the acid-catalyzed reaction between 3-O-acetyl-5′-O-pivaloyluridine (XVIa)[22] and 2,3-dihydro-4*H*-pyran could be selectively de-acetylated with methanolic ammonia to give a mixture of both diastereoisomers of 2′-O-tetrahydropyranyl-5′-O-pivaloyluridine (XVa). The two compounds, which had different TLC mobilities, were separated by silicic acid chromatography and isolated in good yield; they were both obtained crystalline (m.p's 176–178° and 76–78°). The fact that the high-melting diastereoisomer (XVa) and the product obtained directly from the high-melting diastereoisomer of 2′-O-tetrahydropyranyluridine (XIVa) were identical not only establishes the orientation of XVa but also illustrates the comparative selectivity of the reaction between pivaloyl

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* By analogy with the acetylation of thymidine.[30]

chloride* and 2'-O-tetrahydropyranyluridine (XIVa). Further support for the orientation of XVa was provided by the observation that it was quantitatively converted to 5'-O-pivaloyluridine by 80% acetic acid.

The high-melting diastereoisomer of 2'-O-tetrahydropyranyladenosine (XIVb) was similarly converted into its 5'-O-pivaloyl derivative (XVb). The latter compound, which was isolated by silicic acid chromatography in 63% yield, could not be induced to crystallize. However, its consititution which is firmly based on its elemental composition and on its quantitative conversion to 5'-O-pivaloyladenosine,[22] has been confirmed by its use in dinucleoside phosphate synthesis (see below).

Before the 2'-O-tetrahydropyranyl-5'-O-pivaloyl derivatives (XV) were utilized in a dinucleoside phosphate synthesis, it was clearly necessary to discover the conditions required for the removal of each of the protecting groups. It was especially important to find the mildest possible conditions for the hydrolysis of the 2'-O-tetrahydropyranyl function, and thereby minimize any concomitant acid-catalyzed migration of the internucleotidic linkages[32] in the final stage of an oligoribonucleotide synthesis.

Other workers in this field have removed tetrahydropyranyl groups by hydrolysis with aqueous acetic acid.[10, 11, 15] Presumably in order to dissolve intermediate trityl derivatives, Khorana *et al.* used 80% acetic acid and found[10] that removal of the 2'-O-tetrahydropyranyl functions required several hours at room temperature, thereby allowing phosphoryl migration to occur to a significant extent. The half-times of hydrolysis of 2'-O-tetrahydropyranyluridine (XIVa) in 80% acetic acid are 14 and 69 min at 37° and 20°, respectively (Table 2). If the rate of hydrolysis of a tetrahydropyranyl group from a similarly protected dinucleoside phosphate were the same (see below), then ca. 1–2% of phosphoryl migration would be expected to occur[6] in the time required to liberate the 2'-OH group completely. In their earlier work, Smrt and Šorm *et al.*[11] used lower concentrations of acetic acid but often at elevated temperatures. However, in some cases, the acetal and ketal functions which were used[11, 33] by these workers to protect the terminal 2',3'-*cis*-diol

TABLE 2. HALF-TIMES OF HYDROLYSIS OF 2'-O-TETRAHYDROPYRANYL-RIBONUCLEOSIDES[a] (XIV)

Nucleoside	Acidic solvent	Temp	Half-time (min)
Uridine	80% acetic acid	37°	14
Uridine	80% acetic acid	20°	69[b]
Uridine	0·01N HCl	22°	67
Uridine	0·1N HCl	22°	4
Adenosine	0·01N HCl	25°	~60

[a] The high-melting diastereoisomers were used in these studies.

[b] This result was provided by Mr. R. Saffhill.

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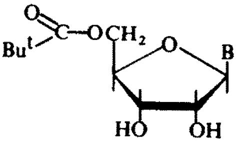
* Selective reaction between pivaloyl chloride and the 5'-hydroxyl function of thymidine has been previously reported.[31]

systems, were appreciably more resistant to acidic hydrolysis than the tetrahydropyranyl group. Thus the extent of phosphoryl migration observed did not directly indicate the limitations of the latter protecting group.* We found that the hydrolysis of 2'-O-tetrahydropyranyluridine (XIVa) displayed first-order kinetics at 22° in both 0·01 N- and 0·1 N-HCl, and that the half-times of reaction were 67 and 4 min, respectively (Table 2). Therefore at 22° and ca. pH 2, the extent of hydrolysis of of 2'-O-tetrahydropyranyluridine† should exceed 99% in under 8 hr. It was not anticipated that appreciable phosphoryl migration would occur under these conditions.



XVII

a; B = uracil-1
b; B = adenine-9

5'-O-Pivaloyl derivatives were found to be much more resistant to base-catalyzed hydrolysis than the corresponding acetates. Thus both 5'-O-pivaloyl-uridine and -adenosine (XVIIa and XVIIb, respectively) were hydrolyzed by methanolic ammonia at inconveniently slow rates. However, the half-time of hydrolysis for both compounds in 15% aqueous methanolic (1:1) methylamine was ca. 8 hr at 20° and, in aqueous methylamine of the same concentration, the half-time of hydrolysis of 5'-O-pivaloyluridine (XVIIa) was ca. 3 hr at 20°. Not unexpectedly, aqueous methanolic tetraethylammonium hydroxide proved to be much more effective and brought about complete removal of the pivaloyl protecting group within 2–3 hr at room temperature; this reagent has been used in all the work described below.

As already indicated, in order that an intermediate protected dinucleoside phosphate may be extended from its 5'-OH end, it is necessary that its terminal 2',3'-diol system should, like the 2'-OH group vicinal to the internucleotidic linkage, be protected with an acid-labile function. Furthermore, if the latter OH group is to be exposed to acidic conditions for the least possible time in the final step of the synthesis, then the protected *cis*-diol system should be at least as acid-labile as the 2'-protecting group. In anticipation of this problem, 2',3'-O-methoxymethylidene nucleosides were prepared[35, 36] and found to undergo acidic hydrolysis at a greater rate than the corresponding 2'-O-tetrahydropyranyl derivatives; the former have been converted into their 5'-phosphates[36] (e.g. XIX) which are required in route (b) oligoribonucleotide synthesis.

The use of 2'-O-tetrahydropyranyl-5'-O-pivaloyluridine (XVIII) as an intermediate in the synthesis of uridylyl-(3' → 5')-uridine (XXI) is illustrated in Chart 2.

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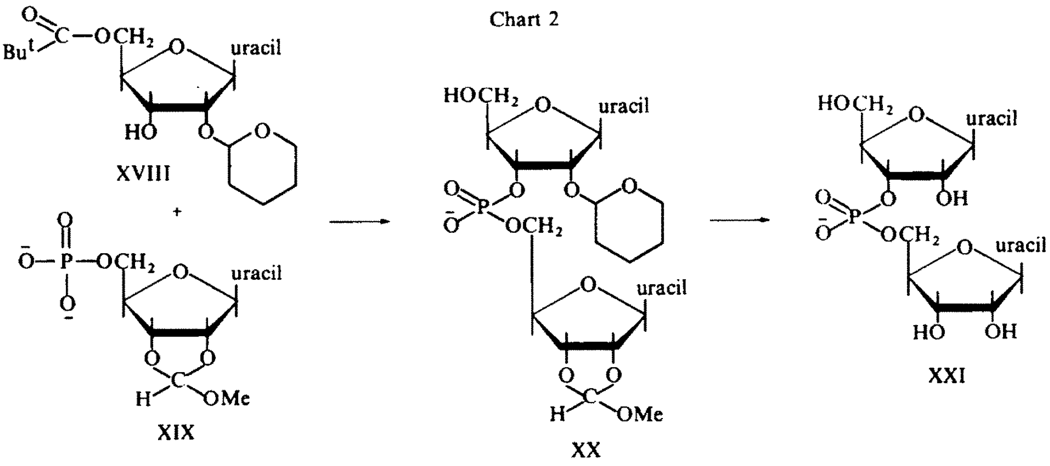
* In a very recent paper[34] on dinucleoside phosphate synthesis, the Czechoslovakian workers have used groups which are more acid-labile than tetrahydropyranyl to protect both the 2',3'-*cis*-diol and 2'-hydroxyl functions. Nevertheless, in two cases, these workers have treated the products with 80% acetic acid for considerable periods (up to 11½ hr) at room temperature to remove the protecting groups.

† A similar rate of hydrolysis was observed for 2'-O-tetrahydropyranyladenosine (XIVb) [see table 2].

The pyridinium salt of 2',3'-O-methoxymethylideneuridine 5'-phosphate (XIX) and a tenfold excess of N,N'-dicyclohexylcarbodiimide were allowed to react with the nucleoside component (XVIII)* in anhydrous pyridine solution at 20°. After 72 hr, the reaction mixture was worked up in the usual manner (Experimental), treated with aqueous methanolic tetraethylammonium hydroxide, and the products then applied to a DEAE-cellulose (bicarbonate form) anion-exchange column. The partially protected dinucleoside phosphate (XX) was eluted in 78% yield (estimated spectrophotometrically) and isolated as an ammonium salt. When this material† was treated with 0·01 N-HCl at 20° and the products neutralized with dilute ammonia, uridylyl-(3' → 5')-uridine (XXI) as obtained as the sole UV-absorbing product. The latter, which had paper chromatographic and electrophoretic properties identical to those of authentic uridylyl-(3' → 5')-uridine, was quantitatively hydrolyzed to uridine 3'-phosphate and uridine in the presence of pancreatic ribonuclease.[37] The low-melting diastereoisomer of 2'-O-tetrahydropyranyl-5'-O-pivaloyluridine was similarly converted into uridylyl-(3' → 5')-uridine, thus establishing beyond doubt the relationship between the diastereoisomers of XVIII (see above).

Chart 2



On the assumption that the rate of hydrolysis of the tetrahydropyranyl group of the partially protected dinucleoside phosphate derivative XX might differ from that of 2'-O-tetrahydropyranyluridine (XIVa), equal quantities of the latter and of the ammonium salt of XX were dissolved together in 0·01 N-HCl and allowed to stand at 24°. After suitable intervals of time, aliquots of the reaction solution

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* 1 mole equiv [with respect to the 5'-nucleotide component (XIX)] of the high-melting diastereoisomer of 2'-O-tetrahydropyranyl-5'-O-pivaloyluridine was added initially, and a further 0·3 mole equiv after 24 hr. A small quantity of Dowex-50 (pyridinium form) cation-exchange resin was added to the pyridine to remove possible trace quantities of aliphatic amines.[14]

† As well as being the synthetic precursor of uridylyl-(3' → 5')-uridine, the partially protected dinucleoside phosphate (XX) is a suitable intermediate for the preparation of dinucleotides with 5'-monophosphate end groups, and also of trinucleoside diphosphates. The phosphorylation of this and other protected dinucleoside phosphates will be considered in the following paper of this series.

were removed, neutralized with dilute aqueous ammonia and separated by a combination of paper electrophoresis and chromatography into starting materials[*] and products, which were estimated spectrophotometrically. It can be seen from the results, plotted in Fig. 1, that the half-time of hydrolysis of the tetrahydropyranyl group from the protected dinucleoside phosphate XX (29 min) is approximately one-half that of the nucleoside derivative XIVa (54 min). This difference is possibly due to the participation of the internucleotidic phosphodiester function in the hydrolysis of the former.

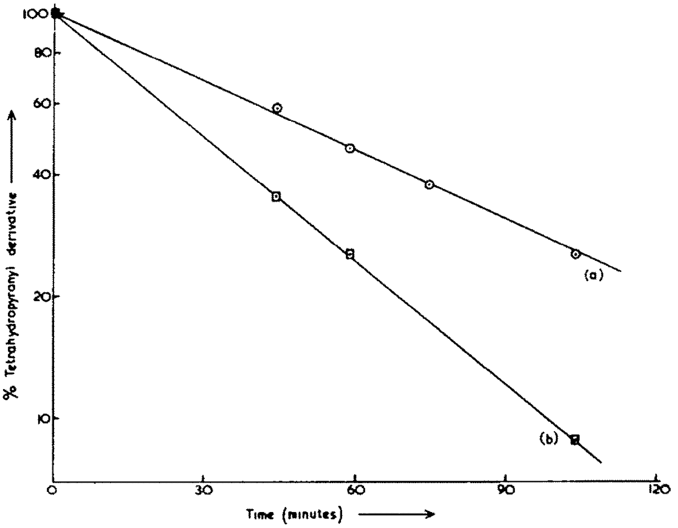

FIG. 1 Hydrolysis of 2′-O-tetrahydropyranyl derivatives in 0·01N-HCl at 24°. (a) —○—○—○— Ordinate indicates percentage of unhydrolyzed 2′-O-tetrahydropyranyluridine; (b) —□—□—□— Ordinate indicates percentage of unhydrolyzed 2′-O-tetrahydropyranyluridylyl-(3′ → 5′)-uridine.

The conditions necessary for the complete removal of the 2′-O-tetrahydropyranyl group from XX in 0·01 N-HCl are so mild (ca. 3–4 hr at room temp) that the extent of concomitant phosphoryl migration is immeasurably small. In order to obtain an estimate of the latter, the partially protected dinucleoside phosphate XX was allowed to react with 0·01 N-HCl for 216 hr at 25°, and the neutralized products then treated with pancreatic ribonuclease. Examination of the resulting digest by paper electrophoresis revealed that only ca. 1% of undegraded dinucleoside phosphate remained. It follows that migration of the internucleotidic linkage, to give pancreatic ribonuclease-resistant[37] uridylyl-(2′ → 5′)-uridine (XXII), occurred

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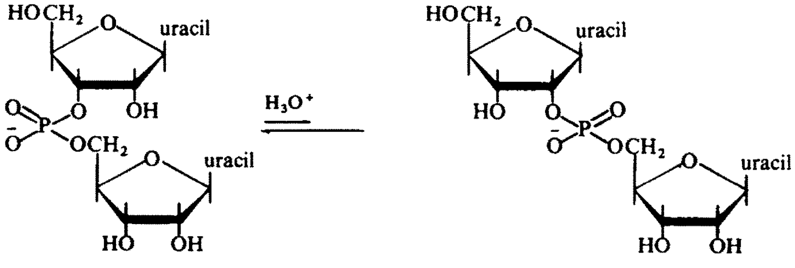
[*] The conversion of 2′-O-tetrahydropyranyluridylyl-(3′ → 5′)-2′,3′-O-methoxymethylideneuridine (XX) into uridylyl-(3′ → 5′)-uridine (XXI) involves the hydrolysis of both the methoxymethylidene and tetrahydropyranyl groups. The half-time of hydrolysis of 2′,3′-O-methoxymethylideneuridine in 0·01N HCl is 10·5 min,[36] and thus the methoxymethylidene group in (XX) would be expected to be ca. 95% hydrolyzed in the shortest reaction time (44 min) recorded in figure 1b. Thus the starting materials in this context are 2′-O-tetrahydropyranyluridine (XIVa) and 2′-O-tetrahydropyranyluridylyl-(3′ → 5′)-uridine. The rate-determining step in the hydrolysis of (XX) is the removal of the tetrahydropyranyl group.

to the extent of 1% in 216 hr. Therefore not more than 0·02% phosphoryl migration* would be expected to occur in the time required for the hydrolysis of the 2′-O-tetrahydropyranyl group. This very small effect may be neglected, and it may be concluded that tetrahydropyranyl is a very satisfactory group for the protection



XXI

XXII

of 2′-OH functions in oligoribonucleotide synthesis. The opposite conclusion was reached in laboratories[10, 15] where 80% acetic was used instead of 0·01 N-HCl: these reagents promote the hydrolysis of the 2′-O-tetrahydropyranyl group at virtually equal rates (Table 2), but the former catalyzes phosphoryl migration at a considerably greater rate† than the latter.

When uridylyl-(3′ → 5′)-uridine (XXI) was treated with dilute hydrochloric acid, hydrolytic cleavage to uridine and uridine 2′(3′)-phosphates was observed as well

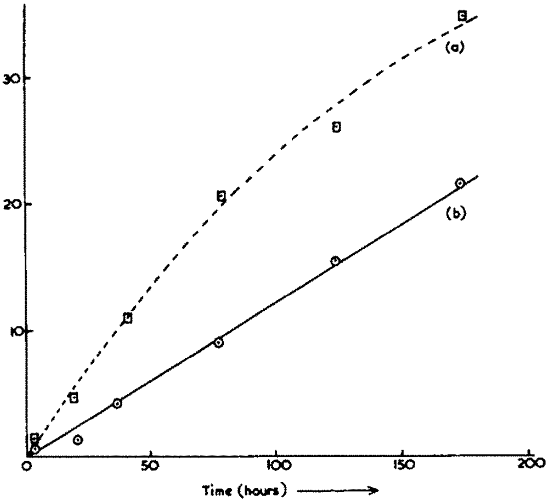


FIG. 2 Hydrolysis and isomerization of uridylyl-(3′ → 5′)-uridine in 0·1N-HCl at 25°. (a) - - - □ - - - □ - - - □ - - - Ordinate represents percentage hydrolysis to uridine and uridine 2′(3′)-phosphates; (b) —○—○—○— Ordinate represents percentage uridylyl-(2′ → 5′)-uridine content of undegraded dinucleoside phosphate.

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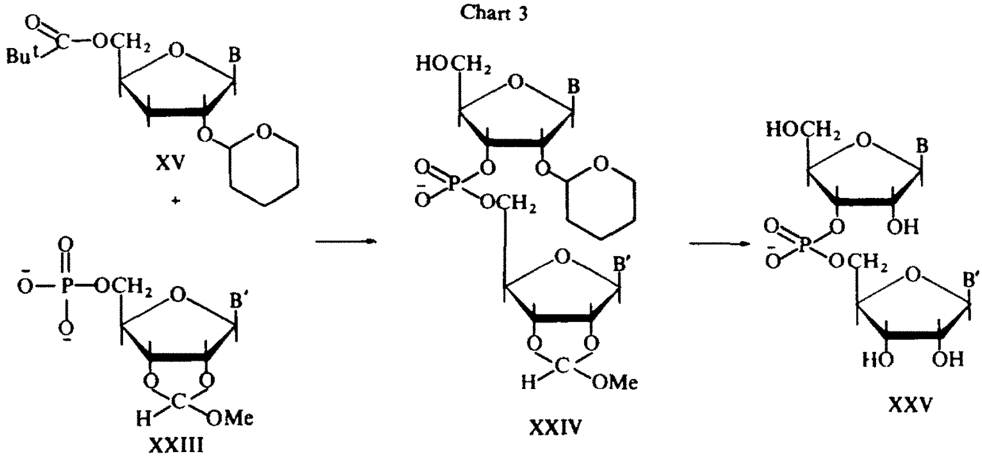
* At constant pH, phosphoryl migration would be expected to proceed by a first-order equilibration reaction.[38] However, the concentration of XXII is so low under these conditions that the reaction would be expected to display simple first-order kinetics.

† From a combination of our and other workers' data,[6] an estimate of the ratio of these rates of ca. 10^2 may be obtained.

as phosphoryl migration:[32] the extent of this degradation in 0·01N-HCl at 25° was only ca. 0·5% in 216 hr. It should be emphasized that while it is generally likely to be very difficult to separate oligoribonucleotides from their 2′ → 5′ isomers the former may readily be freed from their degradation products by anion-exchange chromatography.

In order to obtain a better quantitative estimate of the acid-catalyzed hydrolysis and isomerization of uridylyl-(3′ → 5′)-uridine, the action of 0·1N-HCl on the partially protected dinucleoside phosphate (XX) at 25°, was investigated. The results obtained are illustrated in Fig. 2. Hydrolysis (curve a) appears to follow first order kinetics,* but surprisingly the uridylyl-(2′ → 5′)-uridine content of the undegraded dinucleoside phosphate seems to increase linearly with time, at least in the region examined (curve b). If the rates of hydrolysis of 2′-O-tetrahydropyranyluridine (XIVa) in 0·01 and 0·1N-HCl are compared (Table 2), the time required for the complete removal of the 2′-O-tetrahydropyranyl group from XX may be estimated to be ca. 15–20 min in the latter more concentrated acid at room temperature. It can be seen from Fig. 2 that a negligible amount of degradation and isomerization would occur in this time.

Chart 3



The generality of the present approach to the synthesis of dinucleoside phosphates is illustrated in Chart 3. It should be noted that the partially protected dinucleoside phosphates (XXIV) are also intermediates in the preparation of dinucleotides and trinucleoside diphosphates. Although only 2′-O-tetrahydropyranyl-5′-O-pivaloyl-uridine and -adenosine (XVa and XVb, respectively) are described in this paper, corresponding derivatives of cytidine and guanosine have recently been obtained.[39] The preparation of the required 2′,3′-O-methoxymethylidene-ribonucleoside 5′-phosphates (XXIII) has been reported previously.[36]

The utility of 2′-O-tetrahydropyranyl-5′-O-pivaloyladenosine (XVb) as an intermediate in oligoribonucleotide synthesis was demonstrated by the preparation of

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* If log[dinucleoside phosphate] is plotted against time, a straight line is obtained. As the undegraded dinucleoside phosphate contains appreciable quantities of uridylyl-(2′ → 5′)-uridine (XXII) within 50–100 hr, it follows that the rates of hydrolysis of the latter and uridylyl-(3′ → 5′)-uridine are the same.

ApU* (XXV; B = adenine-9, B′ = uracil-1). The condensation between XVb and 2′,3′-O-methoxymethylideneuridine 5′-phosphate (XXIII; B′ = uracil-1) and the work-up of the products were carried out in the manner described above, in the preparation of UpU. The partially protected dinucleoside phosphate (XXIV; B = adenine-9, B′ = uracil-1) was isolated in 58% yield (Table 3) and hydrolyzed to give ApU, which was quantitatively degraded to adenosine 3′-phosphate and uridine in the presence of spleen phosphodiesterase.[40] In this way, the orientation of 2′-O-tetrahydropyranyl-5′-O-pivaloyladenosine (XVb) was confirmed.

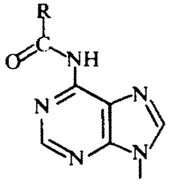
TABLE 3. PREPARATION OF DI-RIBONUCLEOSIDE PHOSPHATES

Dinucleoside phosphate	Hydrolytic enzyme[a]	% Yield of intermediate (XXIV)[b]	Acidic hydrolysis conditions[c] Normality	Time (hr)
UpU	RNase	78	0·01	6
ApU	SP	58	0·01	6
UpA	RNase	61	0·003	24
UpC	RNase	45	0·01	6
ApC	VP	49	0·01	6

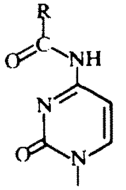
[a] Pancreatic ribonuclease, spleen phosphodiesterase and *Crotalus adamanteus* snake venom phosphodiesterase are abbreviated to RNase, SP and VP, respectively. In each case, quantitative degradation to the appropriate nucleoside and nucleoside 3′(or 5′)-phosphate was observed.

[b] Yields of partially protected dinucleoside phosphates (XXIV), isolated by chromatography on DEAE-cellulose, were estimated spectrophotometrically.

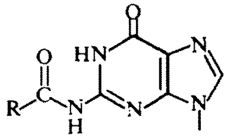
[c] Solutions of the intermediates XXIV in hydrochloric acid (of given normality) were allowed to stand at 20° for the time indicated, and then neutralized. In no case did a detectable amount of the intermediate XXIV remain.



XXVI



XXVII



XXVIII

It is noteworthy that a satisfactory yield of ApU was obtained without protecting the adenine moiety of the nucleoside component (XVb). It has been suggested that, in order to minimize N-phosphorylation, it is desriable to work with acylated adenine[10] (XXVI), cytosine[12] (XXVII), and guanine[41] (XXVIII) residues. Although this procedure has been followed with cytidine derivatives, it is not clear that it is necessary for adenosine derivatives. However, the adenine residue was protected with a *p*-anisoyl group (as in XXVI; R = *p*-MeO—C_6H_4) in the preparation of

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* It is convenient to abbreviate adenylyl-(3′ → 5′)-uridine to ApU. In general, XpY denotes a dinucleoside phosphate in which the 3′-hydroxyl group of nucleoside X and the 5′-hydroxyl group of nucleoside Y are esterified in the phosphodiester internucleotidic linkage. Thus uridylyl-(3′ → 5′)-adenosine is abbreviated to UpA. The nucleosides adenosine, uridine, cytidine, and guanosine are respectively denoted by A, U, C, and G.

UpA from high-melting diastereoisomer of 2′-O-tetrahydropyranyl-5′-O-pivaloyluridine (XVa) and 2′,3′-O-methoxymethylidene-N^6-*p*-anisoyladenosine 5′-phosphate (XXIII; B′ = N^6-*p*-anisoyladenine-9). The condensation and the work-up were conducted in the usual manner,* and the partially protected dinucleoside phosphate (XXIV; B = uracil-1, B′ = adenine-9) isolated by chromatography on DEAE-cellulose, in 61% yield. This yield is comparable with that of the corresponding derivative of ApU (Table 3), despite the fact that the adenine residue was unprotected during the preparation of the latter. The UpA, obtained as the sole UV-absorbing product of acidic hydrolysis of the intermediate XXIV (B = uracil-1, B′ = adenine-9), was completely degraded to uridine 3′-phosphate and adenosine in the presence of pancreatic ribonuclease.

Finally, 2′,3′-O-methoxymethylidene-N^4-benzoylcytidine 5′-phosphate (XXIII: B = N^4-benzoylcytosine-1) was condensed in turn with both 2′-O-tetrahydropyranyl-5′-O-pivaloyl-uridine and -adenosine (XVa and XVb, respectively). These reactions led to satisfactory yields of the protected intermediates (XXIV; B = uracil-1 or adenine-9, B′ = cytosine-1) and hence of the dinucleoside phosphates, UpC and ApC (Table 3).

It is clear from the above results that the route (b) approach to the synthesis of di-ribonucleoside phosphates is satisfactory, and that the tetrahydropyranyl group is suitable for the protection of 2′-OH functions. The partially protected dinucleoside phosphates (XXIV) appear to be completely stable under the conditions required for their purification by DEAE-cellulose chromatography, and readily undergo acid-catalyzed hydrolysis to give the desired products with a negligible amount of isomerization and degradation. It would therefore appear to be unnecessary to seek a 2′-protecting function which is more labile to acid than the tetrahydropyranyl group, 2′-O-acyl protecting groups would seem to be less generally suitable in oligoribonucleotide synthesis as they are appreciably labile under the normal conditions of DEAE-cellulose chromatography,[3] and the use of them can lead to degradation during the final removal of N-acyl protecting groups.[41] In the above work, we have avoided the use of trityl and other hydrophobic functions which would have diminished the solubility of the intermediates in water and thereby complicated the fractionation procedures.

As well as favouring the use of an acid-albile 2′-protecting group in oligoribonucleotide synthesis, we also believe that the route (b) has several advantages over the route (a) approach (Chart 1). In addition to being more fundamental from the point of view of ribonucleoside chemistry, the former approach is also completely general. The methods of preparation of 2′,5′-protected ribonucleoside derivatives, described above, depend neither on the availability of large quantities of nucleoside 2′(3′)-phosphates from natural sources, nor on enzymatic procedures for the specific cleavage of ribonucleoside 2′,3′-cyclic phosphates. Therefore the route (b) approach may readily be used in the synthesis of oligoribonucleotides containing minor bases. The route (a) approach has the added complication that it may sometimes involve the use of non-crystalline (and thus possibly impure) 3′-nucleotide derivatives, and the protection of the latter under conditions which promote phosphoryl migration. Furthermore, the nucleophilic character of the phosphomonoester function may

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* Except that the condensation products were first treated with methanolic ammonia to remove the *p*-anisoyl group.

lead to complications in the protection procedures. In the route (b) approach, the 2′-protected intermediates are pure crystalline compounds, and so usually are the 2′,5′-protected derivatives.

In conclusion, it may be mentioned that the route (b) approach has a further advantage in that both 5′- and 3′-monophosphate intermediates may be used as the phosphorylating species in the synthesis of oligoribonucleotides larger than dinucleoside phosphates. This and other aspects of the subject will be discussed in the subsequent papers of this series.

EXPERIMENTAL

UV absorption spectra were measured with a Cary recording spectrophotometer, model 14M-50. NMR spectra were measured with a Perkin Elmer spectrometer, operating at 60 Mc/s, with TMS as internal standard.

Paper electrophoresis on Whatman No. 4 paper (unless stated otherwise) was conducted with the following buffers: 0·1M-sodium phosphate, pH 6·4; 0·1M-sodium phosphate, pH 8; 0·1M-triethylammonium bicarbonate, pH 7·5; 0·1M-sodium borate, pH 8·0–9·0.

The following solvent systems were used for paper chromatography: A, butan-1-ol–AcOH–water (4:1:5); B, butan-1-ol–AcOH–water (5:2:3); C, propan-2-ol–ammonia (*d* 0·88)–water (7:1:2); D, ethanol–M-aqueous ammonium acetate (5:2); E, butan-1-ol–water (86:14); F, propan-1-ol–ammonia (*d* 0·88)–water (11:2:7); G, isobutyric acid–N-ammonia-0·1M EDTA (100:60:1·6). Ascending chromatograms were run on Whatman No. 1 paper, unless stated otherwise.

Microscope studies, coated with Merck Kieselgel GF_{254}, were used for TLC. The chromatograms were developed with solns of MeOH in $CHCl_3$. Mallinckrodt analytical grade silicic acid (100 mesh) was used for adsorption chromatography.

Pyridine and 2,3-dihydropyran were dried by heating with CaH_2, under reflux, and redistilled before use. Dioxan was similarly dried over $LiAlH_4$, and redistilled.

Preparation of diastereoisomers of 2′-O-tetrahydropyranyluridine (XIVa)

3′,5′-Di-O-acetyluridine[22] (2·0 g) and toluene-*p*-sulphonic acid, monohydrate (0·085 g, 0·08 mol equiv) were stirred with 2,3-dihydropyran (5 ml) and dioxan (15 ml) at 20°. After 30 min, the resulting homogeneous soln was carefully neutralized with M-methanolic MeONa, and then concentrated under reduced press to a viscous gum, which was triturated with $CHCl_3$ (3 × 20 ml). The $CHCl_3$ extracts were filtered through Hyflo-Supercel and evaporated to give a pale-coloured oil, which was dissolved in methanolic ammonia (half-saturated at 0°; 10 ml). The resulting soln was allowed to stand at 20° for 16 hr and was then evaporated under reduced press. The residue was dissolved in $CHCl_3$ (10 ml) and the soln applied to a column of silicic acid (14 cm × 1·3 cm^2). Elution with $CHCl_3$ (ca. 1 l.) gave a non-nucleosidic oily material and a trace of a product with R_f* 0·67. The major nucleosidic products, which had R_f's 0·59 and 0·48, were eluted with 1% MeOH–$CHCl_3$ and 2% MeOH–$CHCl_3$, respectively.

The material with R_f 0·59 (0·85 g, 42%) crystallized from AcOEt to give *the low melting diastereoisomer of 2′-O-tetrahydropyranyluridine* as colourless needles m.p. 146–148°. (Found: C, 51·0; H, 6·3; N, 8·5. $C_{14}H_{20}N_2O_7$ requires: C, 51·2; H, 6·15; N, 8·5%.) UV absorption in water: λ_{max} 262 (log ε 4·09), λ_{min} 229 mμ (log ε 3·33); $[\alpha]_D^{23} = +106°$ (*c* 2, in H_2O). NMR spectrum in dimethylcyanamide–10% D_2O: τ 1·96, doublet, weight 1, assigned to H(6); τ 4·10, doublet (*J* = 3·6 c/s), weight 1, assigned to H(1′); τ 4·31, doublet, weight 1, assigned to H(5); τ 5·19, multiplet, weight 1, assigned to tetrahydropyranyl acetal proton. R_f (system C), 0·82.

The material with R_f 0·48 (also 0·85 g, 42%) crystallized from EtOH to give *the high-melting diastereoisomer of 2′-O-tetrahydropyranyluridine*, m.p. 185–187°. (Found: C, 51·4; H, 6·4; N, 8·9%.) UV absorption in water: λ_{max} 262 (log ε 3·98), λ_{min} 231 mμ (log ε 3·37); $[\alpha]_D^{23} = -47°$ (*c* 1, in H_2O). NMR spectrum in dimethylcyanamide—10% D_2O: τ 2·05, doublet, weight 1, assigned to H(6); τ 3·95, doublet (*J* = 5·1 c/s), weight 1, assigned to H(1′); τ 4·26, doublet, weight 1, assigned to H(5); τ 5·19, multiplet, weight 1, assigned to tetrahydropyranyl acetal proton. R_f (system C), 0·78.

Both diastereoisomers of 2′-O-tetrahydropyranyluridine were found to be free from 3′-isomers by the methanesulphonylation procedure.[42]

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* In this context, R_fs refer to TLC in the solvent system: $CHCl_3$–MeOH (85,15; v/v).

Preparation of diastereoisomers of 2'-O *tetrahydropyranyladenosine* (XIVb)

(a) 3',5'-Di-O-acetyladenosine[22] (1·75 g), toluene-*p*-sulphonic acid, monohydrate (1·04 g, 1·1 mol equiv) and No. 5A Molecular Sieves ($\frac{1}{16}$ in., 0·30 g) were stirred with dioxan (16 ml) for 2 hr at 20°. 2,3-Dihydropyran (2·5 ml) was added, and the reactants stirred for 5 min at 20° and then neutralized by the addition of methanolic MeONa. The products were worked up, as above (in the preparation of 2'-O-tetrahydropyranyluridine), to give a yellow, sweet-smelling glass. The latter was dissolved in methanolic ammonia (half-saturated at 0°, 20 ml), allowed to stand at 20° for 16 hr, then evaporated under reduced press and re-dissolved in EtOH (20 ml). The ethanol soln deposited *the high-melting diastereoisomer of 2'-O-tetrahydropyranyladenosine* (0·875 g, 50%), m.p. 199–201° (Found, in material dried *in vacuo* over P_2O_5 at 100°: C, 51·7; H, 6·1; N, 20·1. $C_{15}H_{21}N_5O_5$ requires: C, 51·3; H, 6·0; N, 19·9%.) UV absorption in water: λ_{max} 258 (log ε 4·16), λ_{min} 227 mμ (log ε 3·42); $[\alpha]_D^{22} = -130°$ (*c* 1, in 95% EtOH). NMR spectrum in dioxan-D_2O (3:1; v/v): τ 1·74, singlet, weight 2, assigned to H(2) and H(8); τ 3·86 (*J* = 6·6 c/s), doublet, weight 1, assigned to H(1'). R_f 0·66 (system C), 0·87 (system A).

The mother liquors were examined by TLC (in solvent system, $CHCl_3$–MeOH, 85:15; v/v), and found to contain two components with R_f's 0·67 and 0·50. The latter component corresponded with the above crystalline 2'-O-tetrahydropyranyladenosine, and the former with the low-melting diastereoisomer (see below). The two diastereoisomers could be fractionated by chromatography on silicic acid.

(b) 3',5'-Di-O-acetyladenosine (5·56 g), toluene *p*-sulphonic acid, monohydrate (3·30 g, 1·1 mol equiv), 2,3-dihydropyran (8 ml, ca. 5 mol equiv) and dioxan (50 ml) were stirred together at 20°. After 20 min, the resulting soln was carefully neutralized with methanolic MeONa. The products were worked up as above, *but not treated with methanolic ammonia*, then dissolved in the minimum quantity of $CHCl_3$ and applied to a silicic acid column (12 cm × 16 cm²). The material eluted from the column with CH_2Cl_2 (1 l.), CH_2Cl_2–$CHCl_3$ (1:1, 750 ml), $CHCl_3$ (1 l.), 1% MeOH–$CHCl_3$ (1 l.), and 2% MeOH–$CHCl_3$ (200 ml) was discarded. Further fractions (100 ml) eluted with 2% MeOH–$CHCl_3$ were retained: fractions 1–8 were combined, evaporated and the resulting material treated with methanolic ammonia under the usual conditions (see above). The products were concentrated to give a chromatographically homogeneous glass with R_f (TLC in $CHCl_3$–MeOH, 90:10; v/v), 0·47. Fractions 9–13 were found to give increasing proportions of a second component (R_f, 0·33) on de-acetylation, while fractions 14 onwards, which were eluted with 2–3% MeOH–$CHCl_3$ gave only the latter component.

Recrystallization of de-acetylated fractions 1–8 (1·1 g, 20%) from AcOEt gave *the low-melting diastereoisomer of 2'-O-tetrahydropyranyladenosine* as colourless needles (0·70 g), m.p. 171–172·5°. (Found, in material dried *in vacuo* over P_2O_5 at 85°: C, 51·5; H, 6·2; N, 19·7%.) UV absorption in water: λ_{max} 259 (log ε 4·05), λ_{min} 226 mμ (log ε 3·22); $[\alpha]_D^{22} = -8°$ (*c* 5, in 95% EtOH). NMR spectrum in dioxan-D_2O (3:1; v/v): τ 1·69, singlet, weight 1, assigned to H(2); τ 1·74, singlet, weight 1, assigned to H(8); τ 3·89, doublet (*J* = 6·3 c/s), weight 1, assigned to H(1'). R_f (system C), 0·68.

The material obtained from the de-acetylation of fractions 14–29 was combined and recrystallized from EtOH to give the high-melting diastereoisomer of 2'-O-tetrahydropyranyladenosine, (3·05 g, 55%). R_f (system A), 0·87.

5'-O-*Pivaloyluridine*

Pivaloyl chloride (0·64 g, 1·5 mol equiv) was added to a soln of 2',3'-O-isopropylideneuridine[22] (1·0 g, 1 mol equiv) in anhyd pyridine (10 ml), and the reactants stirred at 20° for 18 hr. MeOH (2 ml) was added and, after a further period of 1 hr, the products were evaporated under reduced press. A soln of the residue in CH_2Cl_2 (15 ml) was extracted with water (15 ml), dired ($MgSO_4$), evaporated, and then re-dissolved in F_3CCO_2H (2 ml) and water (0·4 ml). After 20 min the soln was evaporated and then re-dissolved in CH_2Cl_2. The latter soln was applied to a column of silicic acid (6 cm × 3 cm²), which was washed in turn with CH_2Cl_2 (200 ml) and $CHCl_3$ (100 ml). The main component, which was eluted with 2% MeOH–$CHCl_3$, crystallized from EtOAc–cyclohexane to give 5'-O-pivaloyluridine (0·72 g, 66%) as a colourless solid, m.p. 84–87°. (Found: C, 50·9; H, 6·1; N, 8·3. $C_{14}H_{20}N_2O_7$ requires: C, 51·2; H, 6·11; N, 8·5%). UV absorption in 95% EtOH: λ_{max} 260 (log ε 3·99), λ_{min} 230 mμ (log ε 3·47).

2'-O-*Tetrahydropyranyl*-5'-O-*pivaloyluridine* (XVa)

(a) A soln of the high-melting diastereoisomer of 2'-O-tetrahydropyranyluridine (0·328 g) in pyridine (3 ml) was allowed to react with pivaloyl chloride (0·18 g, 1·5 mol equiv) at 20°. After 4 hr, MeOH (0·4 ml) was added and, after a further period of 2 hr, the soln was evaporated under reduced press and the residue dissolved in CH_2Cl_2 (5 ml). After extraction with water (5 ml), the CH_2Cl_2 soln was dried (Na_2SO_4) and applied to a column (12 cm × 2·5 cm^2) of silicic acid, which was eluted first with CH_2Cl_2 (350 ml) and then with $CHCl_3$. The fractions eluted with $CHCl_3$, which had R_f (TLC in $CHCl_3$–MeOH, 94:6) 0·41, were combined and concentrated to a glass which crystallized from AcOEt (3 ml) to give *the high-melting diastereoisomer of 2'-O-tetrahydropyranyl-5'-O-pivaloyluridine* (0·19 g, 47%) as colourless needles, m.p. 174–176°. (Found: C, 54·9; H, 6·9; N, 7·0. $C_{19}H_{28}N_2O_8$ requires: C, 55·3; H, 6·8; N, 6·8%.) UV absorption in 95% EtOH: λ_{max} 262 (log ε 4·05); λ_{min} 231 mμ (log ε 3·44). NMR spectrum in $DCCl_3$: τ 2·62, doublet, weight 1, assigned to H(6); τ 4·03 (J ~ 3·5 c/s), doublet, weight 1, assigned to H(1'); τ 4·30, doublet, weight 1, assigned to H(5). R_f (system C), 0·93.

A soln of this material in 80% AcOH was allowed to stand at 20°. After 16 hr, the products were examined by paper chromatography (system C), which revealed a sole UV-absorbing component (R_f, 0·86). The latter, which consumed periodate, had the same R_f as 5'-O-pivaloyluridine.

(b) 3'-O-Acetyl-5'-O-pivaloyluridine[22] (4·4 g), toluene-*p*-sulphonic acid, monohydrate (0·23 g, 0·1 mol equiv), 2,3-dihydropyran (10 ml) and dioxan (25 ml) were stirred together at 20°. After 10 min, the pale red soln was carefully neutralized with M-Methanolic MeONa, and the products concentrated under reduced press to a viscous oil, which was triturated with $CHCl_3$ (5 × 20 ml). The combined $CHCl_3$ extracts were filtered through Hyflo-Supercel, concentrated, and the residue dissolved in methanolic ammonia (half-saturated at 0°, 40 ml) and allowed to stand at 20°. After 16 hr, the soln was evaporated under reduced press to a glass, which was dissolved in CH_2Cl_2 and applied to a column (30 cm × 1·3 cm^2) of silicic acid. The column was eluted with CH_2Cl_2 (1 l.), CH_2Cl_2–$CHCl_3$ (1:1; 500 ml), and then with $CHCl_3$. The last solvent effected the separate elution of two substances with R_f's (TLC in $CHCl_3$–MeOH, 95:5) 0·43 and 0·33.

The fractions containing the first substance (R_f, 0·43) were concentrated and re-chromatographed on silicic acid (column, 9 cm × 4 cm^2) to remove contaminants. The purified material, was eluted with $CHCl_3$ and obtained as a colourless glass (2·3 g, 46%); it solidified when triturated with pet. ether (b.p. 60–80°). A soln of this material in ether deposited crystals of the low-melting diastereoisomer of 2'-O-tetrahydropyranyl-5'-O-pivaloyluridine, m.p. 76–78°. NMR spectrum in $DCCl_3$: τ 2·40, doublet, weight 1, assigned to H(6); τ 4·10, doublet (J ~ 2·2 c/s), weight 1, assigned to H(1'); τ 4·26, doublet, weight 1, assigned to H(5).

The fractions containing the second substance (R_f, 0·33) were combined and concentrated under reduced press to a viscous glass (2·6 g, 51%), which crystallized from EtOH to give the high-melting diastereoisomer of 2'-O-tetrahydropyranyl-5'-O-pivaloyluridine as thick rods, m.p. 176–178°.

2'-O-*Tetrahydropyranyl-5'-O-pivaloyladenosine* (XVb)

A soln of the high-melting diastereoisomer of 2'-O-tetrahydropyranyladenosine (2·0 g) in pyridine (45 ml) was treated with pivaloyl chloride (0·825 g, 1·2 mol equiv) at 20°. After 8 hr, MeOH (5 ml) was added and, after a further period of 1 hr, the products were concentrated under reduced press to a glass. A soln of the latter material in CH_2Cl_2 (6 ml) was applied to a column (20 cm × 1·2 cm^2) of silicic acid which was eluted with CH_2Cl_2 (1 l.), $CHCl_3$ (1 l.), and then with 1% MeOH–$CHCl_3$. The fractions which were eluted with $CHCl_3$ had R_f (TLC in $CHCl_3$–MeOH, 90:10) 0·75; they were combined and evaporated to give a glass (0·28 g). The fractions which were eluted with 1% MeOH–$CHCl_3$ and had R_f, 0·47 (TLC in $CHCl_3$–MeOH, 90:10) were concentrated and then re-evaporated with 95% EtOH to yield *the more polar diastereoisomer** *of 2'-O-tetrahydropyranyl-5'-O-pivaloyladenosine* as a glass (1·57 g, 63%). (Found: C, 54·45; H, 6·9; N, 15·6. $C_{20}H_{29}N_5O_6$, requires: C, 55·15; H, 6·7; N, 16·7%.) R_f (system A), 0·94.

A soln of this material in 0·1 N HCl was allowed to stand at 20°. After 3 hr, the products were examined by paper electrophoresis (borate buffer, pH 9), which revealed a single anionic component with the same mobility as that of 5'-O-pivaloyladenosine.[22]

Preparation of DEAE-cellulose columns for anion-exchange chromatography.

DEAE-cellulose (Bio-Rad CELLEX D medium capacity) is suspended in water (ca. 20 vol), allowed to settle, and the fine particles removed by decantation. This process is repeated several times. It is then suspended in M-triethylammonium bicarbonate (pH 7·0–7·5; ca. 20 vol), allowed to settle, and the buffer decanted. The material is then washed with distilled water (5 × 20 vol) in the same way, suspended in 0·001 M-triethylammonium bicarbonate buffer (pH 7·5), and poured, in several batches, onto a chromatography column which is half-filled with the same buffer. The column is fitted with a coarse glass sinter

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* As this material is non-crystalline, it is referred to as the more polar rather than high-melting diastereoisomer.

and a stopcock, and the latter is left open while the suspension of DEAE-cellulose is added and allowed to settle. The packed column* is washed with 0·001M-buffer (5 vol) before use. The mixture to be fractionated is then applied to the column (see below).

General procedure for preparation of diribonucleoside phosphates

The ammonium salt of the appropriate 2',3'-protected nucleoside 5'-phosphate is applied to a Dowex 50 cation-exchange column (pyridinium form), and the required pyridinium salt washed from the column with 10% aqueous pyridine (ca. 5 bed volumes). The eluate is concentrated under reduced press, in a rotatory evaporator, below 30°, and re-evaporated several times with dry pyridine. *Great care is taken to prevent the solution from becoming acidic.* The appropriate 2'-O-tetrahydropyranyl-5'-O-pivaloyl nucleoside (1 mol equiv) is added to the nucloetide component, the mixture re-evaporated several times with dry pyridine, and then stirred with dry Dowex 50 resin (pyridinium form, ca. 0·025 g/ml pyridine)[14] and N,N'-dicyclohexylcarbodiimide (DCC; 10 mol equiv) in pyridine (ca. 1·5 ml/mmole nucleotide component) at 20°, with the exclusion of moisture. After 24 hr, more of the nucleoside component (0·3–0·5 mol equiv) is added to the reactants and, after a further period of 48 hr, water (2 ml/ml pyridine) is added. The products are then stirred for 12 hr, filtered, and the residue washed with 10% aqueous pyridine. The filtrate and washings are extracted with pet. ether (b.p. 40–60°, 1 vol) and then concentrated† to small volume under reduced press, below 30°, in a rotatory evaporator.

The above material is treated with aqueous methanolic (1:1; v/v) tetraethylammonium hydroxide‡ (0·8M, 5 mol equiv with respect to the original nucleoside component), allowed to stand at 20° for 6–8 hr, and then neutralized by the addition of Dowex 50 (pyridinium form) cation-exchange resin. The resin is removed by filtration and washed with 10% aqueous pyridine. The combined filtrate and washings are applied to a DEAE-cellulose (bicarbonate form) column (see above), which is then washed with 0·001–0·002M-triethylammonium bicarbonate buffer (pH 7·5) until the eluate is no longer UV-absorbing.§ The column is then eluted with triethylammonium bicarbonate buffer (pH 7·5, linear gradient from 0–0·05M over 2 l.). Fractions of 25 ml are collected, and their contents examined by UV spectroscopy and by paper electrophoresis (0·1M-phosphate buffer, pH 8).

The desired protected dinucleoside phosphate is normally eluted in the buffer concentration range 0·025–0·035M, and is usually preceded by small amounts of by-products, of undetermined structures. The fractions containing protected dinucleoside phosphate are combined and the yield estimated spectrophotometrically; they are then concentrated under reduced press, in a rotatory evaporator at below 30°, to dryness.‖ The residue is dissolved in dilute aqueous ammonia (0·005M, ca. 5 ml), and the soln lyophilized to give the protected dinucleoside phosphate as a colourless powder.

This material may be used as an intermediate in the preparation of dinucleotides or trinucleoside diphosphates; when a solution of it in 0·01 N-HCl is allowed to stand at 20° for ca. 6 hr, and then neutralized with dilute ammonia, the unprotected dinucleoside phosphate is obtained.

2'-O-*Tetrahydropyranyluridylyl*-(3' → 5')-2'-2',3'-3'-O-*methoxymethoxymethylideneuridine* (XX)

Pyridinium 2',3'-O-methoxymethylideneuridine 5'-phosphate (from 0·34 g, 0·87 mmole of ammonium salt)[36] was allowed to react with the high-melting diastereoisomer of 2'-O-tetrahydropyranyl-5'-O-pivaloyluridine (0·36 g, 0·87 mmole) and N,N'-dicyclohexylcarbodiimide (1·8 g, 8·7 mmole) in pyridine (10 ml), containing Dowex-50 (pyridinium form) cation-exchange resin. After 24 hr, a further quantity of the nucleoside component (0·11 g, 0·27 mmole) was added, and the reaction allowed to proceed as above.

The products were worked up, and applied to a DEAE-cellulose column (60 cm × 4·5 cm^2). Fractions 46–52 (ca. 100 O.D. units at 260 mμ contained the required product and a material with R_f (system D),

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* A suitable quantity of DEAE-cellulose to separate the products of a condensation initially involving 0·3 mmole of nucleotide component was found, when packed, to fill a column of internal diameter, 2·4 cm to a height of *ca.* 40 cm.

† Pyridine is added at intervals throughout this evaporation process and, as above, great care is taken to prevent the pH from falling below 7.

‡ In the case of condensations involving N (or O)-benzoyl or *p*-anisoyl derivatives of nucleosides or nucleotides, the products are treated with methanolic ammonia (half-saturated at 0°) at 20° for 24 hr before they are treated with tetraethylammonium hydroxide.

§ i.e. until pyridine and nucleosidic material are washed from the column.

‖ The pH rises during the final stages of evaporation.

0·92. Fractions 53–70 (average buffer concentration: 0·035M) contained the required product (13,600 O.D. units at 260 mμ; 78%*) as the sole UV absorbing component; these fractions were combined, and the ammonium salt of 2′-O-tetrahydropyranyluridylyl-(3′ → 5′)-2′,3′-O-methoxymethylideneuridine was isolated as described in the general procedure section (see above). R_f: 0·75 (system D), 0·37 (system C). This preparation was repeated, starting with the low-melting diastereoisomer of 2′-O-tetrahydropyranyl-5′-O-pivaloyluridine.

Uridylyl-(3′ → 5′)-uridine[UpU] (XXI)

A portion of the above protected dinucleoside phosphate (derived from either diastereoisomer of 2′-O-tetrahydropyranyluridine) was dissolved in 0·01N-HCl at 20°. After 6 hr, the products were neutralized with dilute ammonia and found to contain a sole UV-absorbing constituent with R_f: 0·35 (system D), 0·34 (system G). This material had a paper electrophoretic mobility (sodium phosphate buffer, pH 8) identical to that of UpU, prepared from 2′-O-benzyluridine.[23]

The material was completely† hydrolyzed, in the presence of pancreatic ribonuclease to give uridine 3′-phosphate and uridine, which were identified by comparison with authentic marker spots in paper chromatographic systems D and G, and also by paper electrophoresis (pH 8).

2′-O-Tetrahydropyranyladenylyl-(3′ → 5′)-2′,3′-O-methoxymethylideneuridine (XXIV; B = *adenine*-9, B′ = *uracil*-1)

Ammonium 2′,3′-O-methoxymethylideneuridine 5′-phosphate[36] (0·152 g, 0·39 mmole) was converted into the corresponding pyridinium salt, which was allowed to react with 2′-O-tetrahydropyranyl-5′-O-pivaloyladenosine (0·17 g, 0·39 mmole) and N,N′-dicyclohexylcarbodiimide (0·80 g, 3·9 mmole) in pyridine (5 ml), containing Dowex-50 (pyridinium form) cation-exchange resin. After 24 hr, a further quantity of the nucleoside component (0·05 g, 0·115 mmole) was added, and the reaction allowed to proceed as above.

The products were worked up and applied to a DEAE-cellulose column (45 cm × 4·5 cm^2). Fractions 37–45 contained a mixture of two UV-absorbing components (324 O.D. units at 260 mμ with R_f's (system D) 0·93, 0·73. Fractions 46–58 (average buffer concentration: 0·025M) contained the material with R_f 0·73 as the sole UV-absorbing constituent; these fractions were combined (5700 O.D. units at 260 mμ; 58%‡) and treated as above to give the ammonium salt of 2′-O-tetrahydropyranyladenylyl-(3′ → 5′)-2′,3′-O-methoxymethylideneuridine.

Adenylyl-(3′ → 5′)-uridine[ApU] (XXV; B = *adenine*-9, B′ = *uracil*-1)

ApU was obtained as the sole UV-absorbing product, from the protected dinucleoside phosphate, by the general procedure described above; it had R_f 0·37 (system D), 0·54 (system G), and the expected electrophoretic mobility (sodium phosphate buffer, pH 8).

The material was completely hydrolyzed, in the presence of calf-spleen phosphodiesterase§ to give adenosine 3′-phosphate and uridine: these hydrolysis products were identified by comparison with authentic marker spots in paper chromatographic systems D and G, and also by paper electrophoresis (pH 8). It was also hydrolyzed completely to uridine 5′-phosphate and adenosine, in the presence of rattlesnake (*Crotalus adamanteus*) venom phosphodiesterase.§

2′-O-Tetrahydropyranyluridylyl-(3′ → 5′)-2′,3′-O-methoxymethylideneadenosine (XXIV; B = *uracil*-1, B′ = *adenine*-9)

Ammonium N^6-*p*-anisoyl-2′,3′-O-methoxymethylideneadenosine 5′-phosphate[36] (0·08 g, 0·138 mmole) was converted into the corresponding pyridinium salt, which was allowed to react with the high-melting diastereoisomer of 2′-O-tetrahydropyranyl-5′-O-pivaloyluridine (0·058 g, 0·138 mmole) and N,N′-dicyclohexylcarbodiimide (0·29 g, 1·4 mmole) in pyridine (5 ml), containing Dowex-50 (pyridinium form) cation-exchange resin. After 24 hr, a further quantity of the nucleoside component (0·016 g, 0·04 mmole) was added, and the reaction allowed to proceed as above. The products were treated with methanolic

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* Based on the nucleotide starting material and on an extinction coefficient of 20,000 at 260 mμ for the product.

† 0·5–1% of uridylyl-(2′ → 5′)-uridine would have been detected.

‡ Based on the nucleotide starting material and on an extinction coefficient of 25,000 at 260 mμ for the product. This is a conservative estimate as the extinction coefficient is likely to be smaller.

§ Supplied by Worthington Biochemical Corp.

ammonia (see appropriate footnote in general procedure section) before the standard treatment with tetraethylammonium hydroxide and subsequent neutralization with Dowex-50 (pyridinium form) cation-exchange resin.

The products were fractionated on a DEAE-cellulose column (35 cm × 4·5 cm^2) according to the general procedure. Fractions 43–50 (ca. 110 O.D. units) contained two by-products [R_f's: 0·80, 0·52 (system C)]. Fractions 51–57 (350 O.D. units at 260 mμ contained a mixture of *p*-anisic acid and the required product. Fractions 58–65 (average buffer concentration: 0·038M) contained the required product (2100 O.D. units at 260 mμ; 61%*) as the sole UV-absorbing component; these fractions were treated as above to give the ammonium salt of 2'-O-tetrahydropyranyluridylyl-(3' → 5')-2',3'-O-methoxymethylidene-adenosine as a colourless powder. R_f: 0·45 (system C).

Uridylyl-(3' → 5')-*adenosine*[UpA] (XXV; B = *uracil*-1, B' = *adenine*-9)

An aqueous soln of the above material (2350 O.D. units at 260 mμ) was applied to a Dowex-1 × 2 (Cl$^-$, 5 cm × 1 cm^2) anion-exchange column, which was washed with water (500 ml). Fractions subsequently eluted with 0·003N-HCl contained the required dinucleoside phosphate;† these fractions were neutralized with LiOH and concentrated to small volume (ca. 5 ml) under reduced press, in a rotatory evaporator below 30°. Saturated ethanolic $CaCl_2$ (0·5 ml) was added, followed by acetone–EtOH (1:2 v/v, 50 ml). The precipitated calcium salt of uridylyl-(3' → 5')-adenosine was collected by centrifugation, washed with acetone–EtOH and then with ether. The material, which was purified by re-precipitation, had a paper electrophoretic mobility (at pH 8) corresponding to that of ApU; it had R_f 0·19 (system C), 0·55 (system G), and was completely hydrolyzed, in the presence of pancreatic ribonuclease, to uridine 3'-phosphate and adenosine, as indicated by paper chromatography in systems C and G.

2'-O-*Tetrahydropyranyladenylyl*-(3' → 5')-2',3'-O-*methoxymethylidenecytidine* (XXIV; B = *adenine*-9, B' = *cytosine*-1)

Pyridinium N^4-benzoyl-2',3'-O-methoxymethylidenecytidine 5'-phosphate,[36] (from 0·20 g, 0·38 mmole of ammonium salt) was allowed to react with 2'-O-tetrahydropyranyl-5'-O-pivaloyladenosine (0·17 g, 0·39 mmole) and N,N'-dicyclohexylcarbodiimide (0·78 g, 3·8 mmoles) in pyridine (10 ml), containing Dowex-50 (pyridinium form) cation-exchange resin, After 24 hr, a further quantity of the nucleoside component (0·085 g, 0·19 mmole) was added, and the reaction allowed to proceed as above.

The products were worked up‡ and applied to a DEAE-cellulose column (45 cm × 4·5 cm^2). Fractions 34–45 (average buffer concentration: 0·025M) contained the required product (λ_{max} 265 mμ; 4500 O.D. units at 262 mμ, 49%§) as the sole UV-absorbing component; these fractions were treated as above to give the ammonium salt of 2'-O-tetrahydropyranyladenylyl-(3' → 5')-2',3'-O-methoxymethylidenecytidine as a colourless powder, R_f: 0·57 (system C). Fractions 46–52, which were examined by paper electrophoresis (pH 8) and by UV spectroscopy, were found to contain a mixture of the above protected dinucleoside phosphate and benzoate ion.

Adenylyl-(3' → 5')-*cytidine*[ApC] (XXV; B = *adenine*-9, B' = *cytosine*-1)

A soln of the above protected dinucleoside phosphate in 0·01N-HCl was allowed to stand for 6 hr at 20°, and was then neutralized. Paper chromatography revealed a sole UV-absorbing product with R_f: 0·16 (system C). This product had the electrophoretic mobility (at pH 8) expected for ApC; it was completely hydrolyzed, in the presence of rattlesnake (*Crotalus adamanteus*) venom phosphodiesterase, to cytidine 5'-phosphate and adenosine. The latter hydrolysis products were identified by comparison with authentic marker spots in paper chromatographic systems C and G, and also by paper electrophoresis (at pH 8).

* Based on the nucleotide starting material and on an extinction coefficient of 25,000 (at 260 mμ) for the product.

† The tetrahydropyranyl and methoxymethylidene protecting groups were hydrolyzed under the conditions of elution (i.e. in 0·003N HCl). After subsequent neutralization, the products of acidic hydrolysis underwent de-formylation to give the unprotected dinucleoside phosphate.

‡ In this experiment, the methanolic ammonia treatment was omitted and the N^4-benzoyl group was removed (together with the 5'-O-pivaloyl group) by reaction with aqueous methanolic tetraethylammonium hydroxide (18 hr at 20°). This procedure is disadvantageous as it is difficult to separate the liberated benzoate ion from the required product.

§ Based on nucleotide starting material and an extinction coefficient of 24,000 (at 262 mμ) for the product.

2'-O-Tetrahydropyranyluridylyl-(3' → 5')-2',3'-O-methoxymethylidenecytidine (XXIV; B = *uracil*-1, B' = *cytosine*-1)

Pyridinium N^4-benzoyl-2',3'-O-methoxymethylidenecytidine 5'-phosphate[36] (from 0·035 g, 0·065 mmole of ammonium salt) was allowed to react with the high-melting diastereoisomer of 2'-O-tetrahydropyranyl-5'-O-pivaloyluridine (0·03 g, 0·073 mmole) and N,N'-dicyclohexylcarbodiimide (0·145 g, 0·7 mmole) in pyridine (2 ml) containing Dowex-50 (pyridinium form) cation-exchange resin. After 24 hr, a further quantity of the nucleoside component (0·01 g, 0·024 mmole) was added, and the reaction allowed to proceed as above.

The products were worked up,* and applied to a DEAE-cellulose column (42 cm × 1·8 cm^2). Fractions 38–43 (average buffer concentration: 0·030M), which were paper chromatographically (systems C and D) and electrophoretically homogeneous (at pH 8) contained the required product (570 O.D. units at 262 mμ, 45%†); these fractions were treated as above to give the ammonium salt of 2'-O-tetrahydropyranyluridylyl-(3' → 5')-2',3'-O-methoxymethylidenecytidine as a colourless powder, R_f: 0·70 (system C), 0·76 (system D).

Fractions 44–49 were examined by paper electrophoresis and found to contain a mixture of the above protected dinucleoside phosphate and benzoate ion.

Uridylyl-(3' → 5')-cytidine[UpC] (XXV; B = *uracil*-1, B' = *cytosine*-1)

UpC was obtained from the protected dinucleoside phosphate, as the sole UV-absorbing product, by the usual procedure; it had R_f: 0·38 (system C), 0·29 (system G), and the expected electrophoretic mobility (at pH 8). The product was completely hydrolyzed, in the presence of pancreatic ribonuclease, to uridine 3'-phosphate and cytidine. These hydrolysis products were identified by comparison with authentic marker spots in paper chromatographic system G and also by paper electrophoresis (at pH 8).

Alkaline hydrolysis of 5'-O-pivaloyl-nucleosides

(a) To a soln to 5'-O-pivaloyl-nucleoside (0·016 g) in MeOH (1·5 ml) was added aqueous $MeNH_2$ (ca. 30%, 1·5 ml) and the reactants allowed to stand at 20°. The products were analyzed by paper chromatography (system C). The half-times of hydrolysis of the adenosine and uridine derivatives were both ca. 8 hr.

(b) The above experiment with 5'-O-pivaloyluridine was repeated except that the substrate was dissolved in water (1·5 ml) instead of methanol. The half-time of hydrolysis was ca. 3 hr.

(c) To a soln of 5'-O-pivaloyl-nucleoside (0·01 g) in MeOH (0·3 ml) was added aqueous tetraethylammonium hydroxide (1·7M, 0·1 ml) and the reactants allowed to stand at 20°. The products were analyzed by paper chromatography (system A). The half-times of hydrolysis of the adenosine and uridine derivatives were both ca. 15 min.

Acidic hydrolysis of 2'-O-tetrahydropyranyluridine

(a) The high-melting diastereoisomer of 2'-O-tetrahydropyranyluridine (0·005 g) was dissolved in 0·01N-HCl (1 ml) at 22°. After suitable intervals of time, aliquots (0·1 ml) were removed and treated with 0·01N aqueous ammonia (0·2 ml). The products were lyophilized and applied to a Whatman No. 31 paper chromatogram, which was then developed in system C. For each hydrolysis time, the areas containing unchanged starting material and uridine were cut out from the chromatogram and eluted separately with 0·1N-HCl. The two components were estimated spectrophotometrically (at 262 mμ) against the blank (eluted from an equal area of the chromatogram). A linear plot of log [% starting material] against time was obtained. The half-time of hydrolysis was found to be 67 min.

(b) The above procedure was repeated in 0·1N-HCl, and the aliquots of hydrolysate were treated with 0·1N aqueous ammonia. A first-order plot was obtained, and the half-time of hydrolysis was found to be 4 min.

(c) The high-melting diastereoisomer of 2'-O-tetrahydropyranyluridine (0·01 g) was dissolved in AcOH (80% by vol, 1 ml) at 37°. After suitable intervals of time, aliquots were applied to a Whatman No. 541 paper chromatogram (the AcOH was evaporated rapidly, in an air-stream, at room temp), which was developed as before. The half-time of hydrolysis was found to be 14 min.

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* The methanolic ammonia treatment was omitted, as in the preparation of the corresponding ApC derivative.

† Based on nucleotide starting material, and on an extinction coefficient of 19,000 (at 262 mμ) for the product.

Acidic hydrolysis of mixture of 2'-O-tetrahydropyranyl-uridine and adenosine

A soln of the high-melting diastereoisomers of 2'-O-tetrahydropyranyluridine (0·001 g) and 2'-O-tetrahydropyranyladenosine (0·001 g) in 0·01N-HCl (1 ml) was allowed to stand at 25°. After suitable intervals of time, aliquots (0·2 ml) were removed and treated with 0·1N aqueous ammonia (0·1 ml). The products were lyophilized, applied to a strip of Whatman No. 541 paper which was submitted to electrophoresis (45 v/cm) in borate buffer (pH 8). The four components resolved were eluted and estimated spectrophotometrically (at λ_{max}). The half-time of hydrolysis was found to be ca. 60 min for both compounds.

Acidic hydrolysis of 2'-O-tetrahydropyranyluridylyl-(3' → 5')-2',3'-O-methoxymethylideneuridine

(a) The ammonium salt of 2'-O-tetrahydropyranyluridylyl-(3' → 5')-2',3'-O-methoxymethylideneuridine (0·01 g) was dissolved in 0·01N-HCl (1 ml) at 23°. After suitable intervals of time, aliquots (0·1 ml) were treated with 0·01N aqueous ammonia (0·2 ml). The products were lyophilized and applied to a Whatman No. 541 paper chromatogram, which was developed in system F. The protected* and unprotected UpU were eluted and estimated spectrophotometrically. The half-time of hydrolysis to UpU was found to be 30 min.

(b) A soln of the ammonium salt of 2'-O-tetrahydropyranyluridylyl-(3' → 5')-2',3'-O-methoxymethylideneuridine (0·002 g) and the high-melting diastereoisomer of 2'-O-tetrahydropyranyluridine (0·002 g) in 0·01N-HCl (1 ml) was allowed to stand at 24°. After suitable intervals of time, aliquots (0·1 ml) were treated with 0·1N aqueous ammonia (0·1 ml), lyophilized, and applied to a strip of Whatman No. 541 paper, which was submitted to electrophoresis (45 v/cm) in borate buffer (pH 8). Uridine and 2'-O-tetrahydropyranyluridine were obtained as separate components, but the nucleotidic constituents (i.e. UpU and 2'-O-tetrahydropyranyluridylyl-(3' → 5')-uridine) were not separated from each other. The electrophoretogram was cut below the region containing the nucleotidic constituents, and then developed as a paper chromatogram in system F. In this way, all 4 components were separated from each other. The half-time of hydrolysis of the starting materials to UpU and uridine were found to be 29 and 54 min, respectively. The first-order plots obtained are illustrated in Fig. 1.

Acid-catalyzed isomerization of uridylyl-(3' → 5')-uridine

(a) Ammonium 2'-O-tetrahydropyranyluridylyl-(3' → 5')-2',3'-O-methoxymethylideneuridine (0·0035 g, ca. 100 O.D. units at 260 mμ) was dissolved in 0·01N-HCl (1 ml) at 25°. After suitable intervals of time, aliquots (0·2 ml) were treated with 0·1N aqueous ammonia (0·1 ml) and then lyophilized. The residue was dissolved in 0·05M tris hydrochloride buffer (pH 7·5, 0·1 ml) containing pancreatic ribonuclease (50 μg), and the soln maintained at 37°. After 12 hr, the products were lyophilized and applied to a strip of Whatman No. 541 paper, which was submitted to electrophoresis (32·5 v/cm) in phosphate buffer (pH 6·4). The undegraded dinucleoside phosphate was estimated by direct comparison with known amounts† of UpU: after 216 hr, it was found to be ca. 1%.

(b) The above experiment was repeated in 0·1N-HCl. The undegraded dinucleoside phosphate (except in the cases where the extent of isomerization appeared to be <3%) was eluted and estimated spectrophotometrically (assuming $\varepsilon = 20{,}000$ at 260 mμ). The results are plotted in Fig. 2.

Acid-catalyzed degradation of uridylyl-(3' → 5')-uridine

(a) The ammonium salt of protected UpU was treated as above (in the isomerization experiment) with 0·01N-HCl, but the neutralized, lyophilized aliquots were applied directly to a strip of Whatman No. 541 paper, which was submitted to electrophoresis in the same manner. Very small quantities of hydrolysis products (uridine and uridine 2'(3')-phosphates) were estimated by direct comparison. Approximately 0·5% degradation was observed after 216 hr.

(b) The above experiment was repeated in 0·1N-HCl. The hydrolysis products (except in the cases where the extent of hydrolysis appeared to be <3%) were eluted and estimated spectrophotometrically. The results are plotted in Fig. 2.

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* In system F, UpU has R_f 0·43 and both the starting material and 2'-O-tetrahydropyranyluridylyl-(3' → 5')-uridine have R_f 0·63.

† Mixtures containing approximately equimolecular proportions of uridine and uridine 5'-phosphate (*ca.* 10 O.D. units at 260 mμ of each component) and known amounts of UpU (0·1–0·6 O.D. units) were applied to the electrophoretogram alongside the enzymic digest under investigation.

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