Studies on Polynucleotides. XXXVI.[1] The Specific Synthesis of $C_{3'}$–$C_{5'}$–Linked Ribooligonucleotides. IX.[2] The Synthesis of Ribodinucleotides Bearing 3′-Phosphomonoester Groups[3]

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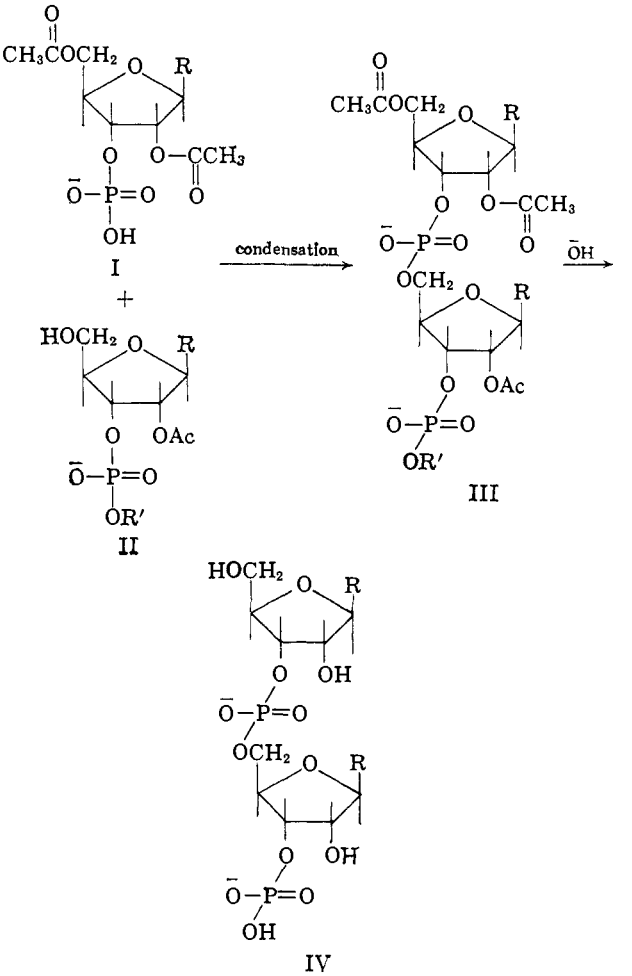
Contribution from the Institute for Enzyme Research of the University of Wisconsin, Madison, Wisconsin. *Received August 6, 1964*

The chemical synthesis of ribodinucleotides bearing 3′-phosphate end groups has been further investigated. The approach used involved (a) the condensation of a protected mononucleotide, e.g., N,O²′,O⁵′-triacetyladenosine 3′-phosphate, with a second suitably protected nucleotide containing an esterified phosphate group, e.g., 1-cyanomethylethyl 2′-O-benzoyluridine 3′-phosphate, and (b) the base-catalyzed removal of the protecting groups. A variety of alcohols whose esters can undergo facile base-catalyzed β-elimination were studied for their suitability for the protection of the phosphate group. 1-Methyl-2-hydroxy-3-butanone was the most suitable. The yields of the dinucleotides using stoichiometric amounts of the two mononucleotide components were between 35 and 40%.

In the preceding paper, three possible approaches to the synthesis of ribodinucleotides bearing 3′-phosphate end groups were discussed, and work on the investigation of two such approaches was described. The present paper records experiments on the use of the third approach which is illustrated by I–IV. While from the practical standpoint, the approach which emerges as the most satisfactory is that which is contained in the preceding paper, the present work is of fundamental interest in connection with the chemistry of the ribonucleotides and in the general problem of the protecting groups in polynucleotide synthesis.

Previously an approach analogous to that shown in I–IV has been developed satisfactorily in the synthesis of deoxyribodinucleotides,[5] the 2-cyanoethyl group serving as the protecting group in one of the nucleotide components. The protecting group was subsequently removed during an alkaline treatment.



I–IV, R = protected or unprotected purine or pyrimidine
II, III, Ac = acetyl or benzoyl; R′ = *e.g.*, $-CH_2CH_2CN$

In the ribonucleotide series a serious restriction on the duration of the alkaline treatment necessary for the removal of such groups (R′ in II and III) is imposed by the free 2′-hydroxyl group. Thus the group, R′, in III should be lost by a facile β-elimination mechanism before any participation from the neighboring 2′-hydroxyl group can occur. The latter type of participation would result in 2′,3′-cyclic phosphate formation and, therefore, the desired dinucleotide (IV) obtained would be contaminated by the isomer containing 2′-phosphomonoester group. The chief problem in the application of the present approach thus lay in the use of an appropriate protecting group (R′) in compounds of type II.

(1) Paper XXXV: D. Söll and H. G. Khorana, *J. Am. Chem. Soc.*, **87**, 350 (1965).

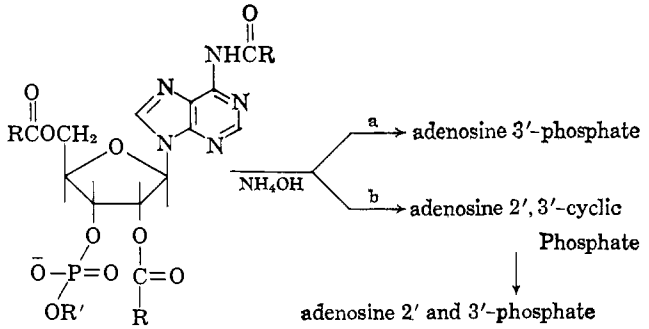
(2) In addition to ref. 1, the following previous papers deal directly with this topic: (a) M. Smith, D. H. Rammler, I. H. Goldberg, and H. G. Khorana, *ibid.*, **84**, 430, (1962); (b) D. H. Rammler and H. G. Khorana, *ibid.*, **84**, 3112 (1962); (c) D. H. Rammler, Y. Lapidot, and H. G. Khorana, *ibid.*, **85**, 1989 (1963); (d) Y. Lapidot and H. G. Khorana, *ibid.*, **85**, 3852 (1963); (e) Y. Lapidot and H. G. Khorana, *ibid.*, **85**, 3857 (1963); (f) C. Coutsogeorgopoulos and H. G. Khorana, *ibid.*, **86**, 2926 (1964); (g) paper XXXIV: R. Lohrmann and H. G. Khorana *ibid.*, **86**, 4188 (1964).

(3) This work has been supported in part by Public Health Service Research Grant No. CA-05178 from the National Cancer Institute, the National Science Foundation Grant No. GB-976, and Life Insurance Medical Research Grant No. G-62-54.

(4) Holder of a Wisconsin Alumni Research Fund Postdoctoral Fellowship.

(5) The condensation of 5′-O-tritylthymidine 3′-phosphate with 2-cyanoethyl thymidine 3′-phosphate gave a good yield of thymidylyl-(3′→5′)-thymidine 3′-phosphate [G. Weimann and H. G. Khorana, *J. Am. Chem. Soc.*, **83**, 419 (1961)]. The synthesis of several dinucleotides bearing 5′-phosphate groups by the condensation of N,O³′-diacyldeoxyribonucleoside 5′-phosphates with nucleoside 5′-2-cyanoethyl phosphate esters has been accomplished: H. Schaller and H. G. Khorana, *ibid.*, **85**, 3841 (1963); E. Ohtsuka, M. Moon, and H. G. Khorana, *ibid.*, in press.

Protecting Groups. Adenosine 2′- and 3′-phosphates can be clearly separated by paper chromatography.[6] Adenylic acid derivatives (general formula V) therefore



V, R = methyl or phenyl, R′ = *e.g.*, cyanoethyl

provided a convenient system for testing the utility of the different protecting groups (R′ in V). Base-catalyzed elimination of the latter and the concomittant removal of the acyl groups would give adenosine 3′-phosphate (route a) whereas attack by the 2′-hydroxyl group while the R′ group is still present would lead to the initial formation of adenosine-2′,3′-cyclic phosphate (route b). The latter would then hydrolyze to give an equal mixture of adenosine 2′- and 3′-phosphates.

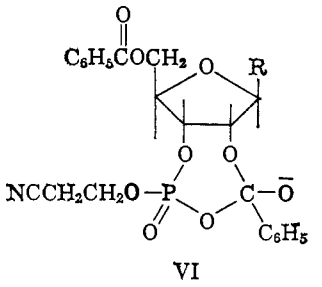
Compounds of the type V were prepared by the reaction of N,O$^{2'}$,O$^{5'}$-triacetyladenosine 3′-phosphate,[2d] or the corresponding tribenzoyl derivative,[2c] with the appropriate alcohol (R′OH) in the presence of DCC followed by purification on DEAE–cellulose anion-exchange columns (see Experimental).

The first compound examined was 2-cyanoethyl N,O$^{2'}$,O$^{5'}$-triacetyladenosine 3′-phosphate (V, R = methyl; R′ = 2-cyanoethyl). The nucleotidic material obtained after appropriate ammoniacal treatment contained about 14% adenosine 2′-phosphate, the remainder being adenosine 3′-phosphate. The next modification sought was to protect the 2′-hydroxyl group in V with the more stable benzoyl group. When 2-cyanoethyl N,O$^{2'}$,O$^{5'}$-tribenzoyladenosine 3′-phosphate (V, R = phenyl; R′ = 2-cyanoethyl) was subjected to ammoniacal treatment, the nucleotide now obtained contained 1.6% of the 2′-phosphate isomer.[7] It is interesting, however, to observe that, as found later, the time (about 45 min.) required for the removal of the 2′-O-benzoyl group during the ammoniacal treatment is only a fraction of that required for the complete removal of the 2-cyanoethyl group (more than 10 hr.). The lesser extent of participation from the 2′-hydroxyl group in the case of the benzoyl derivative than in the case of 2′-O-acetyl derivative can only mean that the greater proportion of the 2-cyanoethyl group was lost at a much higher rate while the 2′-O-benzoyl group was present. This could well be due to a contributing structure such as VI which, by depressing the negative charge on the phosphorus atom, would be expected to accelerate the elimination of the 2-cyanoethyl group.[8]

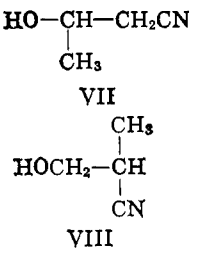
(6) R. Markham and J. D. Smith, *Biochem. J.*, **52**, 552 (1952).

(7) The results are consistent with those reported previously (ref. 2b) on the phosphorylation of N,O$^{3'}$,O$^{5'}$-tribenzoylcytidine with a mixture of 2-cyanoethyl phosphate and dicyclohexylcarbodiimide. After ammoniacal removal of the protecting groups and ion-exchange separation of the cytidylic acid, a single peak corresponding to cytidine 2′-phosphate was observed. This accounted for 95% of the ultraviolet absorbing material applied to the column. In view of the present results, it seems likely that a trace of cytidine 3′-phosphate was also formed but was not detected by the technique.

Dekker and co-workers[9] have recently noted that monoisopropyl uridine 3′-phosphate is extremely stable to alkali. We prepared the isopropyl ester of N-acetyl-



VI



adenosine 3′-phosphate and found it to be likewise stable to alkali or ammonium hydroxide. The use of 1-cyanomethylethyl (corresponding to VII) in place of 2-cyanoethyl for protecting the phosphate group in V was therefore investigated.[10] 1-Cyanopropan-2-ol[11] (1-cyanomethylethyl alcohol), VII, was prepared by the reaction of 1-bromopropan-2-ol[12] with sodium cyanide followed by removal of any trace of the isomeric primary alcohol[11] (VIII) by exhaustive reaction with trityl chloride in pyridine. VII, obtained after distillation, was pure as determined by vapor phase chromatography and by nuclear magnetic resonance spectrum.[13]

1-Cyanomethylethyl N,O$^{2'}$,O$^{5'}$-triacetyladenosine 3′-phosphate (V, R = methyl; R′ = –CH(CH$_3$)(CH$_2$CN)) gave on ammonical treatment the free adenylic acid in which the phosphoryl group had migrated to the extent of 7.3%. This significant migration, although less than that encountered in the corresponding 2-cyanoethyl derivative, must imply that the 2′-hydroxyl group participates in the cyanomethylethyl ester hydrolysis more effectively than in the parent isopropyl ester. However, 1-cyanomethylethyl N,O$^{2'}$,O$^{5'}$-tribenzoyladenosine 3′-phosphate (V, R = phenyl, R′ = –CH(CH$_3$)(CH$_2$CN)) gave on ammoniacal treatment pure adenosine 3′-phosphate.[14]

The use of the 1-cyanomethylethyl group in conjunction with the benzoyl group for the protection of the 2′-hydroxyl group, however, did not prove completely satisfactory for the synthesis of ribodinucleotides. While the time (13 hr. in 7 *N* ammonium hydroxide at room temperature) necessary for the complete removal of these protecting groups in the above-described adenylic

(8) The 2-cyanoethyl group eliminates, in alkali, most slowly from monocyanoethyl phosphate. It eliminates much more rapidly from compounds of the type (RO)(O=)P(OCH$_2$CH$_2$CN)O$^-$ [G. M. Tener, *J. Am. Chem. Soc.*, **83**, 159 (1961)]. The elimination of one of the cyanoethyl groups from neutral esters requires extremely mild conditions (pH 8–9 at room temperature) [H. Schaller and H. G. Khorana, *ibid.*, **85**, 3828 (1963); E. Ohtsuka, M. Moon, and H. G. Khorana, *ibid.*, in press)].

(9) Private communication from Dr. C. A. Dekker, Department of Biochemistry, University of California at Berkeley.

(10) The rate of alkaline elimination of the 1-cyanomethylethyl group was considered to be not very different from that of the cyanoethyl group.

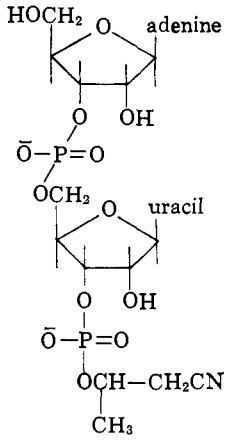
(11) H. S. Davis and B. C. Redmon [(U. S. Patent 2,390,519; *Chem. Abstr.*, **40**, 1870^4 (1946)] who obtained, presumably, a mixture of 1-cyanopropan-2-ol and 2-cyanopropan-1-ol by the ring opening of propylene oxide with hydrogen cyanide, record b.p. 104° (15 mm).

(12) C. A. Stewart and C. A. VanderWerf, *J. Am. Chem. Soc.*, **76**, 1259 (1954).

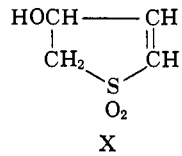
(13) We are grateful to Dr. H. Heyn, University of Chicago, for the determination and interpretation of these spectra.

(14) It should be emphasized that in all these experiments the analysis by paper chromatography was carried out using 0.5–1 μmole of the nucleotide per usual spot so that the 2′-phosphate, if present, would be detected at a concentration of about 1% or less of that of the main product, 3′-phosphate.

acid derivative was relatively safe for the $C_{3'}$–$C_{5'}$-internucleotidic linkage, the corresponding uridylic acid derivatives proved more stable. Thus 1-cyanomethylethyl 2′-O-benzoyluridine 3′-phosphate and 1-cyanomethylethyl adenylyl-(3′→5′)-uridine 3′-phosphate (IX) (see the following section) required about 26 hr. under the same conditions for removal of the cyanomethylethyl group. Prolonged ammoniacal treatment such as this caused considerable breakdown of the internucleotidic linkage in IX. The marked difference observed



IX



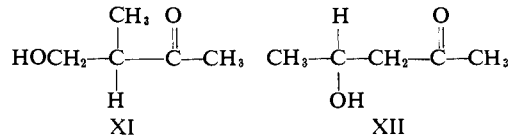
X

in the rate of elimination of the same group present in two different nucleotides illustrates the influence of the structural environment on the rate of removal of the protecting groups.[15] In attempts to accelerate the elimination of the cyanoalkyl groups, the use of divalent ions, such as barium, for the chelation[16] of the phosphate ions was investigated. There was indeed observed an increase (twofold) in this rate in the presence of barium ions, but as was suspected the participation from the 2′-hydroxyl group also increased, and consequently much more (30% in place of 13.5%) of the 2′-nucleotide was produced.

A number of other groups were considered for the protection of the phosphate group in V. 2,2-Dicyanoethyl alcohol appeared attractive but attempts to prepare it by the reaction of 2,2-dichloroethyl alcohol with sodium cyanide or by the addition of formaldehyde to malononitrile[17] failed. The use of 2-nitroethanol apparently gave a diester which was too labile even in aqueous pyridine. Similarly the diester derived from 4-hydroxymethylimidazole[18] was very labile and elimination occurred during purification by column chromatography at pH 7.5 in the cold.

The use of 4-hydroxy-2-sulfolene (X) for esterification of the phosphoric acid group has been described, the ester having a higher rate of elimination than that of the 2-cyanoethyl group.[19] Esters of this alcohol were therefore prepared from N,O2′,O5′-triacetyl- and N,O2′,O5′-tribenzoyladenosine 3′-phosphate. While the rate of elimination was higher, a much higher extent of migration of the phosphoryl group was also observed. This result and the result described above with 1-cyanomethylethyl ester show that the nature of the substituent on the phosphate group influences markedly the participation from the neighboring hydroxyl group.

Finally, alcohols bearing a carbonyl function in the 2-position were investigated. The nucleotide esters derived from some members of this group (*e.g.*, aldol, 2-acetylethyl alcohol) were too labile but those derived from 2-acetyl-2-methylethyl alcohol (1-hydroxy-2-methyl-3-butanone, XI) and 1-methyl-2-acetylethyl alcohol (2-hydroxy-4-pentanone, XII) did have adequate stability. The elimination process in these compounds required about 30 min. at room temperature in 7 *N* ammonium hydroxide. No phosphoryl group migration was detected in the N,O2′,O5′-



XI XII

tribenzoyladenosine 3′-phosphate esters of XI and XII during the removal of the protecting groups. However, the N,O2′,O5′-triacetyladenosine 3′-phosphate derivative of XI showed 14.5% isomerization.

From the total of above studies it is concluded that in the synthesis of ribodinucleotides bearing 3′-phosphate end groups, the use of XI and XII for the protection of the 3′-phosphomonoester group is satisfactory, provided a benzoyl group is present on the 2′-hydroxyl group. It should be added that several of the groups investigated here are potentially useful for work in the deoxyribopolynucleotide field where the problem of phosphoryl group migration during alkaline treatment is virtually absent.

The Synthesis of Ribodinucleotides. A few selected experiments carried out to assess the yield of the ribodinucleotides by the approach illustrated by I–IV may be described. The condensation of N,O2′,O5′-triacetyladenosine 3′-phosphate with the model nucleotide ester, isopropyl 2′-O-acetyluridine 3′-phosphate, was investigated using equimolar proportions of the two nucleotide components. Using DCC as the reagent, the yield of the dinucleotide, isopropyl adenylyl-(3′→5′)-uridine 3′-phosphate, was 35%.[20] With triisopropylbenzenesulfonyl chloride[21] as the reagent, yield of the desired product was 39%.[22] When in the above condensation isopropyl 2′-O-acetyluridine 3′-phosphate was replaced by the corresponding 2′-O-benzoyl derivative, the yield (40%) was again similar. In all these experiments a small amount of adenylyl-(3′→5′)-uridine 3′-phosphate was also produced, presumably by the removal of the isopropyl group during the reaction in anhydrous pyridine.[23]

The condensation of N,O2′,O5′-triacetyladenosine 3′-

(15) Many examples of this kind have been encountered previously. See, *e.g.*, ref. 2c and A. F. Turner and H. G. Khorana, *J. Am. Chem. Soc.*, **81**, 4651 (1959). Similarly, in the present work (Table II) it was found that the elimination of the 2-cyanoethyl group in 2-cyanoethyl thymidine 5′-phosphate occurred at much higher rate than that of the same group in adenosine 3′- and uridine 3′-phosphate derivatives.

(16) See, *e.g.*, K. Dimroth, H. Witzel, W. Hülsen, and H. Myrbach, *Ann.*, **620**, 94 (1959); H. F. Westheimer, Special Publication No. 8, The Chemical Society, London, 1957, p. 55; D. Lipkin, W. H. Cook, and R. Markham, *J. Am. Chem. Soc.*, **81**, 6198 (1959).

(17) The failure in this case was probably due to the elimination of water and subsequent polymerization.

(18) T. C. Bruice and T. H. Fife, *J. Am. Chem. Soc.*, **83**, 1124 (1961).

(19) J. Zemlicka and J. Smrt, *Collection Czech. Chem. Commun.*, 27 2404 (1962).

(20) This includes 4% of the dinucleotide without the isopropyl group.

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

(22) This includes 7% of the dinucleotide without the isopropyl group.

(23) This type of degradation is evident to different extents in polynucleotide synthesis. See, *e.g.*, T. M. Jacob and H. G. Khorana, *J. Am. Chem. Soc.*, 87, 368 (1965).

phosphate with 1-cyanomethylethyl 2′-O-benzoyluridine 3′-phosphate in the presence of DCC followed by ammoniacal treatment (7 *N* at room temperature) gave adenylyl-(3′→5′)-uridine 3′-phosphate and its 1-cyanomethylethyl ester in the total yield of 19%. The principal reason for the reduced yield in this experiment was the prolonged ammoniacal treatment which was necessary for the removal of the 1-cyanomethylethyl group. From the separate determinations of the rates of hydrolysis of the interribonucleotidic linkage in the dinucleotides it was clear that only about 65% of the initially formed product would have survived.

As indicated above, subsequent work showed XI and XII to be the preferred protecting groups. Further experiments to form ribodinucleotides using their nucleotide derivatives were however not pursued because of the success obtained in the generally simpler approach described in the preceding paper.

Included in the Experimental (Table III) is a comparative study of the rate of removal of the 2′-O-acyl groups in uridine 3′-phosphate and isopropyl uridine 3′-phosphate. As expected, the acetyl group was about one-sixth as stable as the benzoyl group and the latter about one-half as stable as the anisoyl group. It was further shown that the 2′-O-acyl group when adjacent to a phosphodiester linkage is more stable than when next to a phosphomonoester group. It is reasonable to expect that the neighboring phosphomonoester group catalyzes the hydrolysis of the acetyl group by forming an acetyl–phosphate anhydride intermediate.

Experimental

The general methods and solvents for paper chromatography and paper electrophoresis were as described in the preceding paper.[1] The R_f's on paper chromatograms and the paper electrophoretic mobilities of the different compounds are recorded in Table I.

1-Cyanopropan-2-ol. A mixture of methyl alcohol (100 ml.), water (45 ml.), and sodium cyanide (37.5 g.) was heated under reflux until a clear solution resulted. 1-Bromopropan-2-ol[12] (100 g.) was added dropwise over a period of 4 hr. and the heating under reflux was continued for a further period of 7 hr. After cooling, the crystalline precipitate was removed by filtration and the filtrate concentrated *in vacuo* to remove methanol and water. The precipitate which appeared was removed and the liquid distilled over a small Vigreux column under reduced pressure. 1-Cyanopropan-2-ol distilled at 102–105° (12 mm.) as a colorless liquid (32.3 g., 52%).[11] This product was dissolved in dry pyridine (35 ml.) and the solution treated with trityl chloride (20 g.) for 4 hr. at 100°. Aqueous ammonium hydroxide (35 ml. of 15%) was added and, after removal of the precipitate, the mixture was distilled over a Vigreux column under reduced pressure. 1-Cyanopropan-2-ol (20.6 g.) was thus obtained as a colorless liquid at 104–108° (12 mm.) (n^{25}D 1.4261). Vapor phase chromatography and the nuclear magnetic resonance spectrum showed it to be pure and free from the isomer,[13] 2-cyanopropan-1-ol. The product gave a 3,5-dinitrobenzoate (m.p. 94–96°, crystals from 60% aqueous ethyl alcohol) which was analyzed.

Anal. Calcd. for $C_{11}H_9N_3O_6$ (279.2): C, 47.32; H, 3.25; N, 15.05. Found: C, 47.12; H, 3.31; N, 15.13.

Table I. Paper Chromatography and Paper Electrophoresis of Different Compounds

Compound	Electrophoretic mobility[a] pH 7.1	pH 2.7	R_f Solvent A	B
5′-O-Dimethoxytrityl-2′-O-benzoyluridine 3′-phosphate	0.36			0.88
2′-O-Benzoyluridine 3′-phosphate	0.76	0.81	0.27	0.59
2′-O-Anisoyluridine 3′-phosphate	0.72		0.37	0.59
5′-O-Dimethoxytrityl-2′-O acetyluridine 3′-phosphate	0.56			0.76
Isopropyl uridine 3′-phosphate	0.51	0.92	0.51	0.77
Isopropyl 2′-O-acetyluridine 3′-phosphate	0.50	0.89		0.83
Isopropyl 2′-O-benzoyluridine 3′-phosphate	0.44			0·82
1-Cyanomethylethyl 2′-O-benzoyluridine 3′-phosphate	0.44			0.82
Isopropyl N-acetyladenosine 3′-phosphate	0.45			0.77
2-Cyanoethyl N,O²′,O⁵′-triacetyladenosine 3′-phosphate	0.48			0.78
1-Cyanomethylethyl N,O²′,O⁵′-triacetyladenosine 3′-phosphate	0.45			0.81
2-Sulfolen-4-yl N,O²′,O⁵′-triacetyladenosine 3′-phosphate	0.42			0.83
2-Acetyl-2-methylethyl N,O²′,O⁵′-triacetyladenosine 3′-phosphate	0.47			0.85
2-Acetyl-1-methylethyl N,O²′,O⁵′-triacetyladenosine 3′-phosphate	0.49			0.85
2-Cyanoethyl N,O²′,O⁵′-tribenzoyladenosine 3′-phosphate	0.34			0.86
1-Cyanomethylethyl N,O²′,O⁵′-tribenzoyladenosine 3′-phosphate	0.34			0.86
2-Sulfolen-4-yl N,O²′,O⁵′-tribenzoyladenosine 3′-phosphate	0.34			0.85
2-Acetyl-2-methylethyl N,O²′,O⁵′-tribenzoyladenosine 3′-phosphate	0.32			0.89
Isopropyl adenylyl-(3′→5′)-uridine 3′-phosphate	0.73	0.75	0.26	0.44

[a] The mobilities under these columns for the compounds derived from different nucleotides are relative to those of the parent ribonucleoside 3′-phosphate except for the dinucleotide derivative, where the mobility is relative to that of uridine 3′-phosphate.

Pyridinium 5′-O-Dimethoxytrityl-2′-O-benzoyluridine 3′-Phosphate. An anhydrous mixture of pyridinium 5′-O-dimethoxytrityluridine 3′-phosphate (1 mmole) and tetraethylammonium benzoate (10 mmole) was treated with benzoic anhydride (20 mmole) and the clear solution which resulted on warming to 50° was kept at room temperature for 50 hr. Methanol (25 ml.), water (10 ml.), and pyridine (2.5 ml.) were added and the mixture was passed through a column of pyridinium Dowex-50 ion-exchange resin. The column was washed with two bed-volumes of 70% aqueous ethyl alcohol. The total eluate and washings were evaporated and the residue was rendered anhydrous by repeated evaporation of added pyridine. Finally, the residue was dissolved in 15 ml. of dry pyridine and the solution added dropwise

to anhydrous ether (350 ml.) with stirring. The fine white powder of 5′-O-dimethoxytrityl-2′-O-benzoyluridine 3′-benzoyl phosphate was collected and dissolved in dry pyridine (10 ml.). Acetic anhydride (5 ml.) was added and the sealed mixture kept at room temperature for 18 hr. Methanol (5 ml.) was then added under cooling and, after 15 min., the solution was evaporated. To the residue was added 25% aqueous pyridine and, after 6 hr. at room temperature, the solution was evaporated. The residue was rendered anhydrous by evaporation of added pyridine and then taken up in 15 ml. of dry pyridine. This solution was added dropwise to anhydrous ether (350 ml.). The fine white precipitate was collected by centrifugation, washed with dry ether, dried, and stored in the desiccator over potassium hydroxide. Pyridinium 5′-O-dimethoxytrityl-2′-O-benzoyluridine 3′-phosphate (500 mg., 0.62 mmole of monopyridinium salt) thus obtained was contaminated by a trace of pyridinium 2′,5′-di-O-benzoyluridine 3′-phosphate, as shown by paper electrophoresis.

Pyridinium 2′-O-Benzoyluridine 3′-Phosphate. Pyridinium 5′-O-dimethoxytrityl-2′-O-benzoyluridine 3′-phosphate (40 mg., 0.05 mmole) was dissolved in 70% aqueous alcohol (5 ml.) and the solution treated with 5 ml. of Dowex-50 (H^+) ion-exchange resin pre-equilibrated with the same solvent. The mixture was shaken for 20 min. at room temperature, and the resin was then removed by filtration and washed with aqueous pyridine. The combined filtrate and the washing were evaporated and the residue was dried by evaporation of added pyridine. The residue was dissolved in dry pyridine (2 ml.) and the solution was added dropwise to an excess of dry ether. The white precipitate of pyridinium 2′-O-benzoyluridine 3′-phosphate was collected by centrifugation, washed with ether, and dried *in vacuo*; λ_{max} 236 and 258 mμ.

Pyridinium 2′-O-Anisoyluridine 3′-Phosphate. An anhydrous solution of pyridinium 5′-O-dimethoxytrityluridine 3′-phosphate (0.1 mmole), tetraethylammonium anisoate (1 mmole), and anisic anhydride (572 mg., 2 mmoles) in freshly distilled dimethylformamide (1.2 ml.) was kept at room temperature in the dark for 25 days. The solvent was then removed *in vacuo*, and the residue was extracted with dry benzene (two 5-ml. portions), then dissolved in 90% aqueous ethanol (10 ml.) and passed through a pyridinium Dowex-50 column. The eluate was coevaporated with pyridine, the residue taken up in dry pyridine (2 ml.), and the solution added dropwise to anhydrous ether (50 ml.). The fine precipitate of pyridinium 5′-O-dimethoxytrityl-2′-O-anisoyluridine 3′-anisoyl phosphate was dissolved in dry pyridine (2 ml.), acetic anhydride (1 ml.) was added, and the sealed mixture was kept at room temperature for ten days. Methanol (1 ml.) was then added and after 15 min. the excess of methanol and methyl acetate was removed by evaporation. Aqueous pyridine (35%) was added and after 7 hr. at room temperature the mixture was coevaporated with pyridine. The residue was dissolved in dry pyridine (2 ml.) and added dropwise to anhydrous ether (50 ml.). The precipitate of pyridinium 5′-O-dimethoxytrityl-2′-O-anisoyluridine 3′-phosphate was collected by centrifugation and dissolved in 70% aqueous ethyl alcohol (30 ml.). The solution was treated with Dowex-50 (H^+) resin (15 ml. of wet resin, previously equilibrated in the same solvent). The mixture was shaken for 20 min. at room temperature. The resin was removed by filtration and the filtrate rendered anhydrous by evaporation of added pyridine. The residue was dissolved in pyridine and the desired product freed from dimethoxytrityl alcohol by an ether precipitation. The white precipitate was dissolved in dry pyridine and stored as a solution in dry pyridine at −15°. At a pH value of 7 in aqueous buffer, the product had λ_{max} 261 mμ; the ratio of extinctions at 280/260 mμ was 0.43.

Pyridinium 5′-O-dimethoxytrityl-2′-O-acetyluridine 3′-phosphate was prepared from pyridinium 5′-O-dimethoxytrityluridine 3′-phosphate by acetylation with acetic anhydride in the presence of tetraethylammonium acetate similar to the procedure for the preparation of pyridinium 2′-O-acetyluridine 3′-phosphate described in the accompanying paper.[1]

Pyridinium Isopropyl 2′-O-Acetyluridine 3′-Phosphate. Isopropyl alcohol (25 ml.) and dry Dowex-50 pyridinium resin (600 mg.) were added to an anhydrous pyridine solution (5 ml.) of pyridinium 5′-O-dimethoxytrityl-2′-O-acetyluridine 3′-phosphate (490 mg., 0.7 mmole), and the total solution was then treated with DCC (3.12 g., 15 mmole). After 65 hr. at room temperature the solution was evaporated to 5 ml., and then water (5 ml.) added. The excess of DCC was extracted with pentane (two 30-ml. portions). The solution was then freed from all pyridine by coevaporation with water. The residue was dissolved in 50% aqueous ethanol (130 ml.). To this was added Dowex-50 (H^+) resin (30 g.)[24] and the mixture was shaken for 15 min. at room temperature. The solution was filtered, the resin was washed with pyridine, and the combined washings and filtrate were evaporated. The residue was dissolved in pyridine (30 ml.) and dimethoxytrityl alcohol extracted with pentane (two 15-ml. portions). Most of the pyridine was then removed under vacuum, the residue taken up in water, and the solution applied on a DEAE–cellulose (carbonate) column (4 × 46 cm.) at 2°. Elution was carried out with a linear gradient, the mixing vessel containing 4 l. of 0.02 *M* triethylammonium bicarbonate and the reservoir an equal volume of 0.1 *M* triethylammonium bicarbonate. Fractions of 17.5 ml. were collected at 10-min. intervals. Tubes 60–75 were pooled (0.165 mmoles) and evaporated with pyridine. The residue was taken up in water and passed through a small Dowex-50 pyridinium resin column. The eluate was quickly frozen and lyophilized. The fine dry powder of this product was stored in the desiccator over potassium hydroxide.

Pyridinium Isopropyl 2′-O-Benzoyluridine 3′-Phosphate. Isopropyl alcohol (10 ml.) was added to an anhydrous pyridine solution (2.5 ml.) of pyridinium 5′-O-dimethoxytrityl-2′-O-benzoyluridine 3′-phosphate (200 mg., 0.25 mmole) and the total solution was then treated with DCC (2.0 g., 10 mmole). After five days at room temperature water (3 ml.) was added and the excess of DCC extracted with cyclohexane (three 10-ml. portions). Then, isopropyl alcohol was removed under vacuum and the solution freed from all pyridine by coevaporation with water. The residue was dis-

(24) The resin was prepared fresh in the acid form and washed thoroughly on a Büchner funnel with 50% aqueous ethanol and sucked dry for about 0.5 hr.

solved in 70% aqueous ethanol (30 ml.). To this was added Dowex-50 (H^+) resin (20 ml., preequilibrated with 70% aqueous ethanol) and the mixture shaken for 15 min. at room temperature. The solution was filtered, the resin was washed with pyridine, and the combined washings and filtrate were evaporated. The residue was dissolved in pyridine (15 ml.) and dimethoxytrityl alcohol extracted with cyclohexane (three 10-ml. portions). Most of the pyridine was then removed under vacuum, the residue was taken up in water, and the solution was applied on a DEAE–cellulose (carbonate) column (46 × 4 cm.). Elution was carried out with a linear gradient, the mixing vessel containing 1.9 l. of 0.01 *M* ammonium bicarbonate and the reservoir an equal volume of 0.15 *M* ammonium bicarbonate in 10% aqueous ethanol. Fractions of 18 ml. were collected at 8-min. intervals. Fractions 78–89 contained the desired product (0.058 mmole). These were pooled and lyophilized to removed ammonium bicarbonate. The desired product was homogeneous by paper chromatography and paper electrophoresis (Table I).

Pyridinium 1-Cyanomethylethyl 2′-O-Benzoyluridine 3′-Phosphate. An anhydrous pyridine solution (1 ml.) of pyridinium 5′-O-dimethoxytrityl-2′-O-benzoyluridine 3′-phosphate (177 mg., 0.2 mmole), 1-cyanopropan-2-ol (0.4 ml.), and DCC (190 mg.) was kept at room temperature in the dark for 60 hr. Water (1 ml.) was then added and the excess of DCC extracted with pentane (three 3-ml. portions). The solution was freed from all pyridine by coevaporation with water and the residue was dissolved in a mixture of dimethylformamide (1 ml.) and 70% aqueous ethanol (20 ml.). This solution was shaken with 20 ml. of Dowex-50 (H^+) ion-exchange resin for 20 min. to remove the dimethoxytrityl group. The subsequent work-up and column chromatography was as described in the preceding experimen

Pyridinium Isopropyl N-Acetyladenosine and Adenosine 3′-Phosphates. Isopropyl alcohol (5 ml.) was added to an anhydrous pyridine solution (2.5 ml.) of pyridinium N,$O^{2'}$,$O^{5'}$-triacetyladenosine 3′-phosphate (25 mg., 0.05 mmole) and the total solution was then treated with DCC (800 mg.). After 48 hr. at room temperature, water (2 ml.) was added and the excess of DCC extracted with cyclohexane (three 5-ml. portions). Isopropyl alcohol was removed under vacuum, the residue taken up in water (3 ml.), and the insoluble dicyclohexylurea removed by filtration. The filtrate was treated with 1 *N* sodium hydroxide (2 ml.) for 30 min. at room temperature to break down the pyrophosphate and the resulting adenosine-2′,3′-cyclic phosphate. After passing the mixture through a small column of pyridinium Dowex-50 resin, the eluate was applied on a DEAE–cellulose (carbonate) column (2.5 × 28 cm.). Elution was carried out with a linear gradient, the mixing vessel containing 1 l. of water and the reservoir an equal volume of 0.1 *M* triethylammonium bicarbonate. Fractions of 17 ml. were collected at 10-min. intervals. Fractions 30–39 contained isopropyl N-acetyladenosine 3′-phosphate (13.8 μmole) whereas isopropyl adenosine 3′-phosphate (5.1 μmoles) was collected in fractions 42–54. Fractions 30–39 were coevaporated with pyridine and stored in dry pyridine at −15°.

Pyridinium 2-Cyanoethyl N,$O^{2'}$,$O^{5'}$-Triacetyladenosine 3′-Phosphate. To an anhydrous pyridine solution (1 ml.) of pyridinium N,$O^{2'}$,$O^{5'}$-triacetyladenosine 3′-phosphate (110 mg., 0.2 mmole) and freshly distilled hydroacrylonitrile (0.2 ml., 2 mmole) was added DCC (160 mg., about 0.8 mmole). The mixture was kept sealed at room temperature in the dark for 3.5 days. Then, water (1 ml.) was added, the excess of DCC extracted with cyclohexane (three 1-ml. portions), and the insoluble dicyclohexylurea removed by filtration. After standing at room temperature for 30 min., the solution was applied on a DEAE–cellulose (carbonate) column (2.8 × 33 cm.) at 2°. Elution was carried out with a linear gradient, the mixing vessel containing 1.5 l. of 0.03 *M* triethylammonium bicarbonate and the reservoir an equal volume of 0.3 *M* triethylammonium bicarbonate. Fractions of 15 ml. were collected at 15 min. intervals. Fractions 8–15 contained the desired diester (0.17 mmole[25]). The pooled fractions were concentrated by repeated evaporation of added pyridine and the product was stored as a solution in dry pyridine at −15°.

Pyridinium 1-Cyanomethylethyl N,$O^{2'}$,$O^{5'}$-Triacetyladenosine 3′-Phosphate. This was prepared from pyridinium N,$O^{2'}$,$O^{5'}$-triacetyladenosine 3′-phosphate as described above for the 2-cyanoethyl ester, 0.4 ml. of 1-cyanopropan-2-ol being used for 0.2 mmole of the nucleotide. The DEAE–cellulose chromatography was carried out at 2°, the pooled peak (first peak after pyridine from column) of the desired product was evaporated in the presence of pyridine, and the product stored as a solution in dry pyridine at −15°.

Pyridinium 2-Sulfolen-4-yl N,$O^{2'}$,$O^{5'}$-Triacetyladenosine 3′-Phosphate. The diester was prepared by the reaction of pyridinium N,$O^{2'}$,$O^{5'}$-triacetyladenosine 3′-phosphate (0.2 mmole) with 4-hydroxy-2-sulfolene[26] (0.5 ml.) in pyridine (1 ml.) in the presence of DCC (160 mg.). The work-up was as described above for the preceding compound.

Pyridinium 2-Acetyl-2-methylethyl N,$O^{2'}$,$O^{5'}$-Triacetyladenosine 3′-Phosphate. A solution of pyridinium N,$O^{2'}$,$O^{5'}$-triacetyladenosine 3′-phosphate (55 mg., 0.1 mmole), freshly distilled 2-acetyl-2-methylethyl alcohol (1-hydroxy-2-methyl-3-butanone, 0.25 ml.), and DCC (100 mg.) in dry pyridine (0.5 ml.) was kept sealed at room temperature in the dark for 24 hr. Water (0.5 ml.) was then added and, after the extraction of DCC and the removal of dicyclohexylurea, the products were applied to the top of a DEAE–cellulose (carbonate) column (33 × 3 cm.). The elution was performed at 2° with a linear gradient, the mixing vessel containing 750 ml. of 0.02 *M* triethylammonium bicarbonate while the reservoir contained an equal volume of 0.1 *M* triethylammonium bicarbonate. Fractions of 8 ml. at 7.5-min. intervals were collected. Fractions 33–38 contained the desired product (0.06 mmole). The eluent was removed by coevaporation with pyridine and the product stored in dry pyridine at −15°. In aqueous pyridine, the product underwent decomposition. In dry pyridine at

(25) Ammonium N,$O^{2'}$,$O^{5'}$-triacetyladenosine 3′-phosphate (eluted from a chromatogram in solvent B) was deacylated in 7 *N* ammonium hydroxide in a cuvette and the change of spectrum followed with time. The absorption maximum of the N-acetyl compound (273 mμ) quickly disappeared. After 1 hr. the new peak at 259 mμ (adenylic acid) reached its final height. From this experiment was calculated ϵ_{273} 13.4 × 10^3 for N,$O^{2'}$,$O^{5'}$-triacetyladenosine 3′-phosphate.

(26) M. Prochazka and V. Horak, *Collection Czech. Chem. Commun.* **24**, 1509 (1959).

low temperature (−20°), the decomposition was slow but detectable (6% in six weeks).

Pyridinium 2-Acetyl-1-methylethyl N,O²′,O⁵′-Triacetyladenosine 3′-Phosphate. The compound was prepared as described for the isomeric diester in the preceding experiment. The starting alcohol was 2-hydroxy-4-pentanone. The product underwent decomposition on storage (31% in six weeks in dry pyridine at −20°).

Pyridinium 2-Cyanoethyl N,O²′,O⁵′-Tribenzoyladenosine 3′-Phosphate. To an anhydrous pyridine solution (1 ml.) of pyridinium N,O²′,O⁵′-tribenzoyladenosine 3′-phosphate (152 mg., 0.2 mmole) and freshly distilled hydroacrylonitrile (0.25 ml., 2.5 mmole) was added DCC (206 mg., 1 mmole) and the mixture was kept sealed at room temperature in the dark for 20 hr. Water (1 ml.) was then added, the excess of DCC was extracted with cyclohexane (three 1-ml. portions), and the insoluble dicyclohexylurea was removed by filtration. After standing at room temperature for 30 min. the solution was applied on a DEAE-cellulose (carbonate) column (4 × 46 cm.). Elution was carried out with a linear gradient, the mixing vessel containing 1.5 l. of 20% aqueous ethyl alcohol and the reservoir an equal volume of 0.1 *M* triethylammonium bicarbonate in 20% aqueous alcohol. Fractions of 20 ml. were collected at 10-min. intervals. Fractions 118–136 contained the desired diester (1100 $O.D._{260}$). The pooled fractions were coevaporated with pyridine and stored in dry pyridine at −15°.

Pyridinium 1-Cyanomethylethyl N,O²′,O⁵′-Tribenzoyladenosine 3′-Phosphate. This was prepared as described in the preceding experiment for 2-cyanoethyl phosphate ester, 0.4 ml. of 1-cyanopropan-2-ol being used for 0.2 mmole of the protected nucleotide.

Pyridinium 2-Sulfolen-4-yl N,O²′,O⁵′-Tribenzoyladensosine 3′-Phosphate. This was prepared from the tribenzoyl nucleotide (0.2 mmole) and 4-hydroxy-2-sulfolene[26] (2 mmole) using DCC (0.8 mmole) as described above. The product was purified by DEAE-cellulose column chromatography in the standard way.

Pyridinium 2-Acetyl-2-methylethyl N,O²′,O⁵′-Tribenzoyladenosine 3′-Phosphate. The diester was prepared by the reaction of pyridinium N,O²′,O⁵′-tribenzoyladenosine 3′-phosphate (0.023 mmole) with 1-hydroxy-2-methyl-3-butanone (0.2 ml.) in pyridine (0.3 ml.) in the presence of DCC (0.23 mmole). The work-up done at 2° was as described above. The diester obtained was pure as shown by paper electrophoresis and was used immediately for hydrolysis experiments.

Hydrolysis of Different "Alkyl" Esters of Protected Ribonucleoside 3′-Phosphates. Monoalkyl esters of N,O²′,O⁵′-triacetyladenosine 3′-phosphate, N,O²′,O⁵′-tribenzoyladenosine 3′-phosphate, and 2′-O-benzoyluridine 3′-phosphate (15–20 μmoles/ml.) were kept in aqueous 7 *N* ammonium hydroxide at room temperature. Aliquots (equivalent to about 1 μmole of nucleotide) were removed at different intervals and examined by paper electrophoresis at pH 7.1. The starting diesters could thus be separated from the resulting monoesters (nucleotides). For determination of the phosphoryl group migration in the resulting nucleotides, the treatment with ammonium hydroxide was extended to five days to ensure complete breakdown of the possible intermediate nucleoside-2′,3′-cyclic phosphate. The product was examined by paper chromatography in the solvent, aqueous saturated ammonium sulfate–0.1 *M* buffer solution (pH 6)–isopropyl alcohol (79:19:2, v./v.).[6] This solvent clearly separated adenosine 2′- and 3′-phosphates. The extent of isomerization and the rates of elimination of the different "alkyl" groups in ammonium hydroxide were determined by elution of the different spots and of the appropriate blanks followed by spectrophotometric analysis. The results are given in Table II.

Table II. Hydrolysis of Ribonucleoside 3′-Phosphate Esters

Compounds	Hydrolysis of diester bond		Migration, %
	Hours	%	
7 *N* Ammonium hydroxide			
2-Cyanoethyl N,O²′,O⁵′-triacetyladenosine 3′-phosphate	10	>90	13.5
1-Cyanomethylethyl N,O²′,O⁵′-triacetyladenosine 3′-phosphate	13	>90	7.3
2-Sulfolen-4-yl N,O²′,O⁵′-triacetyladenosine 3′-phosphate			38.5[a]
2-Acetyl-2-methyl N,O²′,O⁵′-triacetyladenosine 3′-phosphate[c]	0.5	100	14.5
2-Acetyl-1-methyl N,O²′,O⁵′-triacetyladenosine 3′-phosphate[c]	0.5	100	0.5[b]
2-Cyanoethyl N,O²′,O⁵′-tribenzoyladenosine 3′-phosphate	10	>90	1.6
1-Cyanomethylethyl N,O²′,O⁵′-tribenzoyladenosine 3′-phosphate[c]	13	>90	0
2-Sulfolen-4-yl N,O²′,O⁵′-tribenzoyladenosine 3′-phosphate			23.5[a]
2-Acetyl-2-methylethyl N,O²′,O⁵′-tribenzoyladenosine 3′-phosphate[c]	0.5	100	0
2-Cyanoethyl thymidine 5′-phosphate	7	>90	
1-Cyanomethylethyl 2′-O-benzoyluridine 3′-phosphate	26	>90	
Isopropyl N-acetyladenosine 3′-phosphate	24	<1	
Isopropyl uridine 3′-phosphate	24	<1	
7 *N* Ammonium hydroxide–0.2 *N* barium acetate			
2-Cyanoethyl N,O²′,O⁵′-triacetyladenosine 3′-phosphate	4	>90	30
1-Cyanomethylethyl N,O²′,O⁵′-triacetyladenosine 3′-phosphate	6	>90	21.4
7 *N* Ammonium hydroxide–0.01 *M* lanthanum nitrate			
1-Cyanomethylethyl N,O²′,O⁵′-triacetyladenosine 3′-phosphate	10	>90	5.8

[a] The diester readily gives adenosine-2′,3′-cyclic phosphate, which causes the high degree of isomerization. [b] The detected small amount of adenosine 2′-phosphate is probably due to a slight contamination of the diester with di-(N,O²′,O⁵′-triacetyladenosine-3′)-pyrophosphate. [c] During the hydrolysis of the above esters a minor compound (in the case of the 2-acetyl-1-methylethyl ester, however, about 30% of the total material) was formed. It shows an adenosine spectrum. The electrophoretic mobility (pH 7.1) is 0.39 and the R_f value (solvent A) 0.93.

Hydrolysis of 2′-O-Acyl Group in 2′-O-Acylribonucleoside 3′-Phosphates. About 15 μmoles of each of the different compounds was kept in 7 *N* aqueous ammonium hydroxide (0.5 ml.) at room temperature. Aliquots (0.5–1 μmole) were removed at different intervals and examined by paper electrophoresis at pH 7.1. The time for the complete loss of the 2′-O-acyl group in different compounds is given in Table III.

Cleavage of Internucleotidic Linkage in Ammonium Hydroxide. An aqueous solution (0.15 ml.) of the

Table III. Time (min.) for Complete Removal of 2'-O-Acyl Group in 7 *N* Ammonium Hydroxide at Room Temperature

	Time, min.
2'-O-Acetyluridine 3'-phosphate	5
2'-O-Benzoyluridine 3'-phosphate	35
Isopropyl 2'-O-benzoyluridine 3'-phosphate	50
2'-O-Anisoyluridine 3'-phosphate	70

ribodinucleotide (0.7–1 μmole) was treated with an equal volume of concentrated ammonium hydroxide at room temperature. Aliquots of 0.04 ml. were removed at different intervals and applied on paper chromatograms which were developed in solvent A. The extent of internucleotidic bond cleavage as determined by the disappearance of the starting material and by the concentration of the nucleotides produced was found to be: Adenylyl-(3'→5')-uridine, adenylyl-(3'→5')-uridine 3'-phosphate, cytidylyl-(3'→5')-uridine 3'-phosphate: 46% in 24 hr., 77–79% in 72 hr.; inosinyl-(3'→5')-uridine 3'-phosphate: 50% in 24 hr., 89% in 72 hr.

Condensation of N,O²',O⁵'-Triacetyladenosine 3'-Phosphate and Isopropyl 2'-O-Acetyluridine 3'-Phosphate. (a) Using DCC. A mixture of pyridinium N,O²',O⁵'-triacetyladenosine 3'-phosphate (55 mg., 0.1 mmole), pyridinium isopropyl 2'-O-acetyluridine 3'-phosphate (44 mg., 0.1 mmole), and dry Dowex-50 (pyridinium) resin (200 mg.) was rendered anhydrous by several evaporations of added dry pyridine. The residue was taken up in dry pyridine (2 ml.) and the solution was treated with DCC (300 mg., 1.46 mmole). After thorough mixing some pyridine (about 0.5 ml.) was removed under vacuum. The resulting clear solution containing the resin suspension was shaken for five days at room temperature in the dark. Water (1.5 ml.) was then added and the excess of DCC extracted with pentane (three 5-ml. portions). The insoluble dicyclohexylurea was removed by filtration and the filtrate treated with an equal volume of concentrated ammonium hydroxide for 2 hr. Ammonia was removed by evaporation and the residue made up with water to 10 ml. One-fourth of this solution was applied on top of a DEAE-cellulose (carbonate) column (2 × 22 cm.). Elution was carried out with a linear gradient, the mixing vessel containing 1.5 l. of 0.02 *M* ammonium bicarbonate and the reservoir an equal volume of 0.1 *M* ammonium bicarbonate. Fractions of 9 ml. were collected at 5-min. intervals. The desired isopropyl adenylyl-(3'→5')-uridine 3'-phosphate was eluted off the column together with the mononucleotides. Rechromatography of that peak on paper in solvent A separated the components. The yield of isopropyl adenylyl-(3'→5')-uridine 3'-phosphate was 31%. Some adenylyl-(3'→5')-uridine 3'-phosphate (4%) was also present.

(b) Using Triisopropylbenzenesulfonyl Chloride.[21] An anhydrous pyridine solution (1 ml.) of pyridinium N,O²',O⁵'-triacetyladenosine 3'-phosphate (27 mg., 0.05 mmole) and pyridinium isopropyl 2'-O-acetyluridine 3'-phosphate (22 mg., 0.05 mmole) was treated with triisopropylbenzenesulfonyl chloride (40 mg., 0.13 mmole) under exclusion of moisture.[27] After 12 hr. at room temperature water (0.2 ml.) was added and the mixture was kept at 0° for 12 hr. The subsequent work-up and separation on a DEAE-cellulose column by paper chromatography was as described above. The yield of isopropyl adenylyl-(3'→5')-uridine 3'-phosphate was 32%. Some adenylyl-(3'→5')-uridine 3'-phosphate (7%) was also present.

Condensation of N,O²',O⁵'-Triacetyladenosine 3'-Phosphate and Isopropyl 2'-O-Benzoyluridine 3'-Phosphate. An anhydrous pyridine solution (1 ml.) of pyridinium N,O²',O⁵'-triacetyladenosine 3'-phosphate (19 mg., 0.035 mmole) and pyridinium isopropyl 2'-O-benzoyluridine 3'-phosphate (19 mg., 0.035 mmole) was treated with triisopropylbenzenesulfonyl chloride[21] (40 mg., 0.13 mmole) under exclusion of moisture.[27] A clear solution resulted on shaking for about 15 min. After 6 hr. at room temperature the reaction was terminated by adding 10% aqueous pyridine (0.6 ml.) to the cooled (−70°) solution. The mixture was allowed to warm to room temperature and left for 15 min., then treated with an equal volume of concentrated ammonium hydroxide for 2 hr. Ammonia was then removed by evaporation and the remaining aqueous solution was made up with water to a stock solution of 9 ml., which was kept frozen. Separation by ion exchange and paper chromatography was as described above. The yield of isopropyl adenylyl-(3'→5')-uridine 3'-phosphate was 39%; 1% of adenylyl-(3'→5')-uridine 3'-phosphate was also formed.

Condensation of N,O²',O⁵'-Triacetyladenosine 3'-Phosphate and 1-Cyanomethylethyl 2'-O-Benzoyluridine 3'-Phosphate. A mixture of pyridinium N,O²',O⁵'-triacetyladenosine 3'-phosphate (55 mg., 0.1 mmole), pyridinium 1-cyanomethylethyl 2'-O-benzoyluridine 3'-phosphate (solution in pyridine about 0.04 mmole), and dry Dowex-50 (pyridinium) resin (200 mg.) was rendered anhydrous by several evaporations of added dry pyridine. The residue was taken up in dry pyridine (1.5 ml.) and the solution was treated with DCC (80 mg.) The resulting clear solution containing the resin suspension was kept at room temperature for four days in the dark. Water (1.5) ml. was then added and the excess of DCC extracted with pentane (three 3-ml. portions). The insoluble dicyclohexylurea was removed by filtration and the filtrate made up with 50% aqueous pyridine to 5 ml. One-third of the solution was treated with an equal volume of concentrated ammonium hydroxide. After 18 hr.[28] at room temperature, most of the ammonia was evaporated, and the solution was applied on top of a DEAE-cellulose column, the conditions for chromatography being as described above. A total of 19% of the nucleotidic material recovered from the column was accounted for by adenylyl-(3'→5')-uridine 3'-phosphate (8%) and 1-cyanomethylethyl adenylyl-(3'→5')-uridine 3'-phosphate (11%).

(27) The addition was made inside a drybox.
(28) Paper-electrophoresis at this point showed the incomplete removal of the 1-cyanomethylethyl group.