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THE SYNTHESIS OF OLIGORIBONUCLEOTIDES—V*

THE PREPARATION OF SOME DINUCLEOTIDES AND TRINUCLEOSIDE DIPHOSPHATES

B. E. GRIFFIN and C. B. REESE

University Chemical Laboratory, Cambridge, England

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Abstract Partially-protected derivatives of three di-ribonucleoside phosphates (with free 5'-OH groups) have been converted into the corresponding dinucleotides (pUpU, pUpC and pApC), in good yields. The preparation of 2'-O-tetrahydropyranyl-5'-O-acyl derivatives of both uridine and adenosine 3'-phosphates is described. The trinucleoside diphosphates UpUpU, UpApU and ApApU have been prepared, in satisfactory yields, from the appropriate partially-protected dinucleoside phosphates (with free 5'-OH groups) and the above protected nucleoside 3'-phosphates. All the products described have been synthesized under conditions which ensure that they contain exclusively 3' → 5'-internucleotidic linkages. None of the synthetic steps has involved the use of enzymes

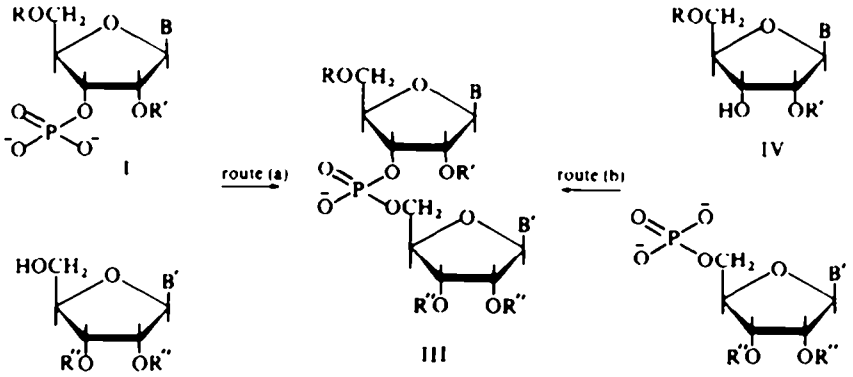
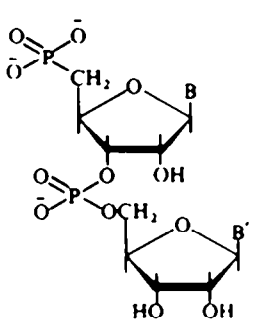


CHART 1

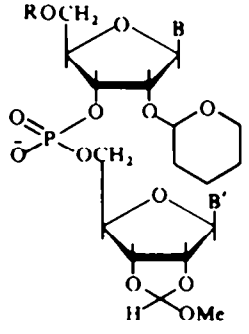
IN THE previous paper of this series,[1] we considered the relative merits of routes (a) and (b) [see chart 1] in the initial step of oligoribonucleotide synthesis. Whereas other workers[2-4] in this field had concentrated on the route (a) approach [i.e. condensation between a 2',5'-protected ribonucleoside 3'-phosphate (I) and a 2',3'-protected ribonucleoside (II)], we investigated the alternative route (b) approach [i.e. condensation between a 2',5'-protected ribonucleoside (IV) and a 2',3'-protected ribonucleoside 5'-phosphate (V)], which we found to be very satisfactory.[1,5,6] We also reached the conclusion that it was most convenient to protect 2'-OH functions, vicinal to internucleotidic phosphodiester linkages, with acid-labile groups and that the tetrahydropyranyl acetal system was suitable for this purpose.[1] The latter group could be removed by acidic hydrolysis, under such mild conditions that the extent of degradation and phosphoryl migration was negligible.

* For part IV of this series, see Ref. 1

The protecting groups were chosen so that the terminal 5'-OH function of III could be liberated selectively. Then the latter derivatives (III) could be intermediates in the preparation not only of dinucleoside phosphates but also of dinucleotides with terminal 5'-monophosphate functions (VI). As it had been decided that R' (see formula III) should be acid-labile, it was necessary that R'' should also be acid-labile and that R should be base-labile. The desired fully-protected dinucleoside phosphates (VIIb), prepared[1] by the condensation between 2',3'-O-methoxymethylidene-ribonucleoside 5'-phosphates[7] and 2'-O-tetrahydropyranyl-5'-O-pivaloyl ribonucleosides,[1,5] were treated with base* to free the terminal 5'-OH functions and thus give the partially-protected intermediates VIIa. The latter have been readily converted, by treatment with dilute aqueous acid, into free dinucleoside phosphates.[1] We now wish to describe their conversion both into dinucleotides (VI) and trinucleoside diphosphates (XII).



VI



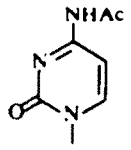
VII

a: R = H
b: R = tBuCO

Initially, the synthesis of uridylyl-(5' → 3')-uridine 5'-phosphate† (VI; B = B' = uracil-1) was undertaken. The appropriate intermediate[1] VIIa (B = B' = uracil-1), two molecular equivalents of β-cyanoethyl phosphate and an excess of N,N'-dicyclohexylcarbodiimide (DCC) were allowed to react together in anhydrous pyridine solution, under the usual conditions.[8] The products were worked up (Experimental), treated with aqueous ammonia to remove the β-cyanoethyl protecting group,[8] and then applied to a Dowex-1 anion-exchange column. An acidic solution (ca. pH 2) was required to elute the products, and consequently the tetrahydropyranyl[1] and methoxymethylidene[7] protecting groups were removed during the fractionation. Since the latter process was completed in under 24 hr, the extent of degradation and isomerization of the desired dinucleotide was negligible:[1] pUpU was obtained in ca. 80% yield, and isolated as a paper chromatographically homogeneous, colourless lithium salt. The structure assigned to this product rests on the observation that it was completely degraded, in the presence of pancreatic ribonuclease, to pUp and uridine. This dinucleotide had previously been prepared by Smrt and Šorm[9] from a different partially-protected UpU derivative.

* The reagents used[1] to effect the removal of the pivaloyl group were aqueous methanolic tetraethylammonium hydroxide and aqueous methylamine. Removal of acyl blocking groups from the pyrimidine and purine base-residues also occurs under these conditions.

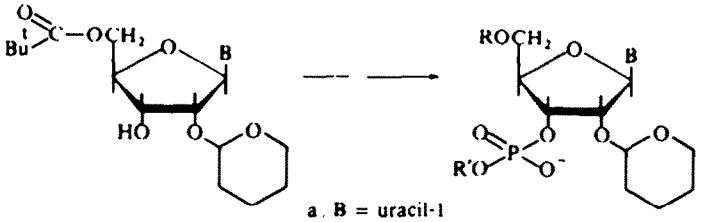
† This dinucleotide may be represented, in an abbreviated form, as pUpU. Uridine, adenosine, cytidine and guanosine are abbreviated to U, A, C and G, respectively. Esterification (with phosphoric acid or a monoalkyl phosphoric acid) of the 5'- and 3'-OH functions of a particular nucleoside is indicated by placing p, respectively, before and after the letter representing that nucleoside.



VIII

By a slightly modified procedure, the dinucleotides pUpC and pApC (VI; B = uracil-1 or adenine-9; B′ = cytosine-1) were both obtained in approximately 60% yields. The modification involved the N^4-acetylation of the cytosine residues (as in VIII) of the appropriate partially-protected dinucleoside phosphates, prior to phosphorylation. Thus, in the preparation of pUpC, the starting material VIIa (B = uracil-1, B′ = cytosine-1) was allowed to react with an excess of acetic anhydride in pyridine solution, and the product* then treated briefly with aqueous sodium hydroxide[10] to give the required N^4-acetyl derivative VIIa (B = uracil-1, B′ = VIII). The conditions for the phosphorylation of the latter and the subsequent ammoniacal treatment† were as described above in the preparation of pUpU. The same modification‡ was adopted in the preparation of pApC from partially-protected ApC (VIIa; B = adenine-9, B′ = cytosine-1). Both the cytidine-containing dinucleotides were purified by chromatography on Dowex-1 resin, with concomitant removal of the acid-labile protecting groups.

Both pUpC and pApC were isolated as colourless, paper chromatographically homogeneous lithium salts; in accord with its assigned structure, the former was completely digested to pUp and cytidine in the presence of pancreatic ribonuclease. However, pApC was stable under the latter conditions, but was cleaved to a mixture of adenosine and cytidine 5′-phosphates when incubated with *Crotalus adamanteus* snake venom phosphodiesterase. It is noteworthy that pApC is one of the dinucleotides which have recently been isolated[11] from *Euchaeta japonica* embryos.



IX a, B = uracil-1 b, B = adenine-9 X

Although it is intended, in our main approach to oligoribonucleotide synthesis, that the chain-extension step should involve the phosphorylation of a 3′-OH function with a 5′-nucleotide derivative [see chart 1, route (b)], it seemed worthwhile to examine the utility of the partially-protected dinucleoside phosphates (VIIa) as intermediates in the synthesis of trinucleoside diphosphates, and possibly even of larger oligomers.

* The product is first treated with aqueous pyridine to cleave possible acetyl phosphate linkages, and give the diacetyl derivative VII (R = Ac, B = uracil-1, B′ = VIII). In NaOH soln, saponification of the 5′-acetate occurs more readily than hydrolysis of the N^4-acetyl function.[10]

† This ammoniacal treatment removes the N^4-acetyl residue, as well as the β-cyanoethyl group.

‡ In this case, the adenine residue may also be acylated.

Chain-extension of these derivatives (VIIa) may be effected by a route (a) approach, involving the phosphorylation of their terminal 5'-OH groups with 2',5'-protected nucleoside 3'-phosphates.* We therefore undertook the preparation of 2'-O-tetrahydropyranyl-5'-O-acyl derivatives of uridine and adenosine 3'-phosphates (respectively, Xa and Xb; R = acyl, R' = H) from the corresponding nucleoside derivatives.

The high-melting diastereoisomer of 2'-O-tetrahydropyranyl-5'-O-pivaloyluridine[1] (IXa) was phosphorylated with β-cyanoethyl phosphate and DCC, in the usual way.[8] Following chromatography of the products on DEAE-cellulose, the β-cyanoethyl ester of 2'-O-tetrahydropyranyl-5'-O-pivaloyluridine 3'-phosphate (Xa; R = tBuCO, R' = CH_2CH_2CN) was isolated in 90% yield. As the aqueous ammoniacal treatment required to eliminate the β-cyanoethyl group also caused considerable de-acylation, the above material (Xa; R = tBuCO, R' = CH_2CH_2CN) was allowed to react with aqueous methanolic tetraethylammonium hydroxide which removed both the β-cyanoethyl and pivaloyl groups to give 2'-O-tetrahydropyranyluridine 3'-phosphate (Xa; R = R' = H). This material was treated with an excess of acetic anhydride in anhydrous pyridine solution to give its diacetyl derivative (Xa; R = R' = Ac), which underwent hydrolysis in aqueous pyridine solution[12] to the desired product, 2'-O-tetrahydropyranyl-5'-O-acetyluridine 3'-phosphate (Xa; R = Ac, R' = H). The latter was isolated as a pyridinium salt, in virtually quantitative yield.† The corresponding analytically pure monoammonium salt was also prepared. This represents the fully chemical synthesis of a compound which had only been obtained previously[13] by a route which involved an enzymatic step.

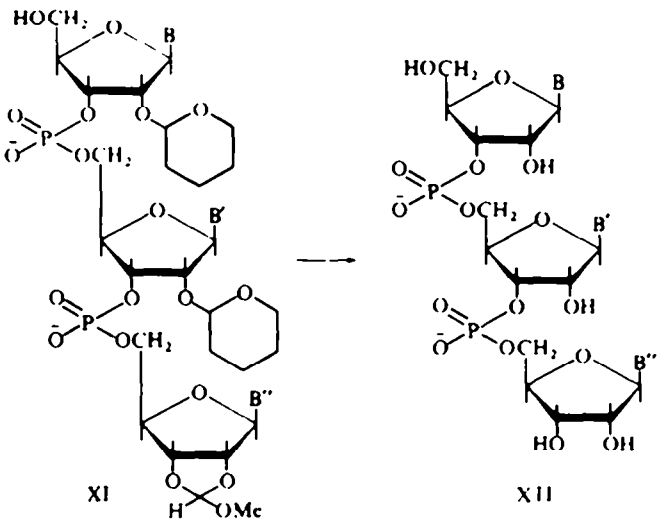
In the same way, 2'-O-tetrahydropyranyl-5'-O-pivaloyladenosine (IXb) was converted into its 3'-β-cyanoethyl phosphate (Xb; R = tBuCO, R' = CH_2CH_2CN) which was also purified by chromatography on DEAE-cellulose and isolated in good yield. When this material was treated with 3N-aqueous ammonia at 50° for 30 min, the β-cyanoethyl group was removed with a very small amount of concomitant de-acylation. Thus the isolated ammonium salt of 2'-O-tetrahydropyranyl-5'-O-pivaloyladenosine 3'-phosphate (Xb; R = tBuCO, R' = H) was contaminated with only ca. 5% of 2'-O-tetrahydropyranyladenosine 3'-phosphate (Xb; R = R' = H). Although it was demonstrated (Experimental) that the latter could be obtained pure following a re-acylation step, the 95% pure material was found to be quite satisfactory for synthetic purposes.

The availability of these 2',5'-protected nucleoside 3'-phosphates (X; R = acyl, R' = H) allowed the synthesis of certain trinucleoside diphosphates to be undertaken. As a first example, partially-protected UpU (VIIa; B = B' = uracil-1), 1·5 molecular equivalents‡ of 2'-O-tetrahydropyranyl-5'-O-acetyluridine 3'-phosphate (Xa; R = Ac, R' = H), and an excess of DCC were allowed to react together in anhydrous dimethylformamide/pyridine solution. Following the usual work-up procedure, the products were treated with methanolic ammonia and then fractionated by chromatography on DEAE-cellulose, using aqueous triethylammonium bicarbonate buffer (pH 7·5)

* This procedure corresponds to that adopted by Smrt *et al.*,[3] except that none of the synthetic steps involves the use of an enzyme.

† Based on 2'-O-tetrahydropyranyl-5'-O-pivaloyluridine 3'-β-cyanoethyl phosphate (Xa; R = tBuCO, R' = CH_2CH_2CN).

‡ The mononucleotide component (⅔) was added initially, and the remaining ⅓ after 24 hr.



XI XII

a B = B′ = B″ = uracil-1
b B = B″ = uracil-1, B′ = adenine-9
c B = B′ = adenine-9, B″ = uracil-1

as the eluting agent. The desired product XIa, which was eluted when the buffer concentration was ca. 0·12M, was obtained in ca. 57% yield, and isolated as a colourless ammonium salt.

To obtain a highly pure specimen of UpUpU, an aqueous solution of this material was applied to a Dowex-1 anion-exchange column, which was then eluted with 0·01N HCl, containing increasing amounts of lithium chloride. As in the above preparation of dinucleotides, the acid-labile protecting groups were removed during the fractionation process. After the fractions containing UV-absorbing material had been neutralized (with LiOH), the free trinucleoside diphosphate, UpUpU (XIIa) was precipitated as a colourless calcium salt, which was paper chromatographically and electrophoretically homogeneous. In a separate experiment, a solution of the partially-protected trinucleoside diphosphate (XIa) in 0·01N HCl was allowed to stand at 20° for 10 hr, then neutralized and the products incubated with pancreatic ribonuclease. Paper chromatography and electrophoresis indicated complete digestion to uridine 3′-phosphate and uridine, thus establishing that the acidic hydrolysis had removed all the protecting groups from XIa to give UpUpU (XIIa), and that the latter contained only 3′ → 5′H internucleotidic linkages.[14]

In the same way, 2′-O-tetrahydropyranyl-5′-O-acetyluridine 3′-phosphate (Xa; R = Ac, R′ = H), partially-protected ApU (VIIa; B = adenine-9, B′ = uracil-1), and an excess of DCC were allowed to react together in anhydrous dimethylformamide–pyridine solution. Initially, stoicheiometric quantities of the two nucleotidic reactants were used but, after 40 hr, more of the dinucleoside phosphate component was added. The products of this reaction were worked-up, treated with methanolic ammonia and fractionated on DEAE-cellulose as before. The desired partially-protected UpApU (XIb), which was eluted with ca. 0·14M aqueous triethylammonium bicarbonate buffer (pH 7·5), was obtained in 54% yield and isolated as a colourless triethylammonium salt.

A solution of the latter (XIb) in 0·01N HCl was allowed to stand at 20° for 8 hr, and was then neutralized with dilute aqueous ammonia to give the free trinucleoside diphosphate, UpApU (XIIb). When this material, which was paper chromatographically and electrophoretically homogeneous, was incubated with *Crotalus adamanteus* snake venom phosphodiesterase, it was completely degraded to uridine 5'-phosphate, adenosine 5'-phosphate and uridine; it was quantitatively digested to Up and ApU in the presence of pancreatic ribonuclease.

Finally, partially-protected ApU (VIIa; B = adenine-9, B' = uracil-1) was allowed to react with 2'-O-tetrahydropyranyl-5'-O-pivaloyladenosine 3'-phosphate (Xb; R = tBuCO, R' = H) and an excess of DCC in dimethylformamide–pyridine solution. Following the procedure adopted in the preparation of UpApU (XIIb), an excess of the dinucleoside phosphate component was used and, as before, it was added in two portions. After the reaction mixture had been worked-up, the products were treated with aqueous methanolic tetraethylammonium hydroxide to hydrolyze the 5'-pivalate function. Fractionation on DEAE-cellulose was carried out according to the usual procedure: the desired partially-protected ApApU (XIc) was eluted with ca. 0·15M triethylammonium bicarbonate buffer (pH 7·5), and obtained in ca. 54% yield. This material was isolated as a colourless triethylammonium salt, which was contaminated with a small quantity (ca. 5%) of another UV-absorbing substance.

The protecting groups were removed from XIc in the manner described for the corresponding UpApU derivative (XIb). The free ApApU (XIIc) so obtained, was further purified by chromatography on DEAE-cellulose and isolated as a colourless, paper chromatographically and electrophoretically homogeneous, triethylammonium salt. When this material was incubated with *Crotalus adamanteus* snake venom phosphodiesterase, it was completely degraded to uridine 5'-phosphate, adenosine 5'-phosphate and adenosine.

Although the partially-protected trinucleoside diphosphates (XI) are suitable intermediates for the preparation of tetranucleoside triphosphates, it is not intended to develop this approach to oligoribonucleotide synthesis further. We have confirmed our earlier conclusion[1,5] regarding the suitability of the acid-labile tetrahydropyranyl group (or some other acetal or ketal system with a similar lability) for the protection of 2'-OH functions, and intend to report shortly on the extension of oligoribonucleotide chains solely by the route (b) approach [see chart 1], which we believe to be more convenient.

EXPERIMENTAL

UV absorption spectra were measured with a Cary recording spectrophotometer, model 14M-50. Paper electrophoresis was usually conducted at 2–4 kV in 0·1M-sodium phosphate buffer (pH 8) on Whatman No. 4 paper. The following solvent systems were used for paper chromatography: A, propan-2-ol-ammonia (*d* 0·88)–water (7:1:2); B, ethanol–M aqueous ammonium acetate (5:2); C, isobutyric acid–N ammonia–0·1 M EDTA (100:60:1·6). Ascending chromatograms were run on Whatman No. 1 paper, unless otherwise stated. Column chromatographic separations were carried out on Bio-Rad Cellex D DEAE-cellulose (medium capacity), prepared for use as described previously[1] Anhyd pyridine (supplied by British Drug Houses, Ltd.) was heated with CaH_2 under reflux, and then fractionated.

2'-O-Tetrahydropyranyl-5'-O-acetyluridine 3'-phosphate (Xa; R = Ac, R' = H).

To an anhyd soln of pyridinium β-cyanoethyl phosphate[8] (2·0 mmoles, from 0·645 g Ba salt) in pyridine (10 ml) was added the high-melting diastereoisomer IXa[1] (0·412 g, 1·0 mmole) and DCC (2·06 g, 10·0 mmoles)

The reaction mixture was sealed and stirred magnetically at 20°. After 2 days, water (10 ml) was added and the reaction mixture stirred for a further 10 hr. The precipitated N,N'-dicyclohexylurea was removed by filtration and washed with 20% aqueous pyridine (5 ml). The combined filtrate and washings were extracted with pet. ether (b.p. 40–60°, 2 × 10 ml), and concentrated to ca. ⅓ volume. Paper electrophoresis (pH 8) of the aqueous layer revealed a principal component (>90% of total UV-absorbing material) with a mobility ca. 0·4 that of uridine 3'-phosphate.

The above soln was applied to a DEAE-cellulose anion-exchange column (HCO_3^- form, 40 cm × 5 cm^2), which was then washed with 0·002M triethylammonium bicarbonate buffer (pH 7·5) until the eluate no longer absorbed in the UV. The column was then eluted with triethylammonium bicarbonate buffer (pH 7·5, linear gradient from 0·002–0·05M over 2 l.). Fractions (25 ml) were collected: fractions 22–35 (average buffer concentration ca. 0·02M) contained the desired β-cyanoethyl ester of 2'-O-tetrahydropyranyl-5'-O-pivaloyluridine 3'-phosphate [8950 O.D. units,* 90% (based on ε_{260} = 10,000)].

The above material (4525 O.D. units) was concentrated† to dryness below 30° under reduced press on a rotatory evaporator, redissolved in tetraethylammonium hydroxide [ca. 0·8M in aqueous MeOH (1:1), 3 ml], and allowed to stand at 20°. After 16 hr, Dowex-50 (pyridinium form) cation-exchange resin was added until the pH dropped to 7. Paper electrophoresis (pH 8) of the products revealed 2'-O-tetrahydropyranyluridine 3'-phosphate as the sole UV-absorbing constituent [R_f 0·31 (system A)] with a mobility ca. 0·95 that of uridine 3'-phosphate.

The resin was removed by filtration, and washed with 10% aqueous pyridine (2 × 10 ml). The combined filtrate and washings were concentrated under reduced press, re-evaporated several times with pyridine, then dissolved in pyridine (10 ml) and treated with Ac_2O (0·5 ml) at 20°. After 16 hr MeOH (5 ml) was added and after a further 2 hr the products were concentrated, under reduced press below 30°, almost to dryness and then re-dissolved in 10% aqueous pyridine (30 ml). After it had been allowed to stand at 5° for 16 hr,[12] this soln was concentrated and then re-evaporated several times with pyridine to yield a pale-coloured oil. The latter was dissolved in pyridine (3 ml) and the soln added dropwise, with stirring, to ether (30 ml). The resulting ppt was collected by centrifugation, washed with ether and dried *in vacuo* over P_2O_5. The yield of pyridinium 2'-O-tetrahydropyranyl-5'-O-acetyluridine 3'-phosphate (0·245 g) was virtually quantitative.

A soln of the above pyridinium salt in 10% aqueous pyridine (5 ml) was passed through a Dowex-50 × 8 (NH_4^+ form) cation-exchange column (5 cm × 1 cm^2), which was then washed with 10% aqueous pyridine (5 bed volumes). The total eluate was concentrated and re-evaporated several times with anhyd pyridine. *Monoammonium 2'-O-tetrahydropyranyl-5'-O-acetyluridine 3'-phosphate* was isolated as a nearly colourless solid by the precipitation technique described above. (Found: N, 8·14; P, 5·55. $C_{15}H_{23}N_3O_{11}P$, $4H_2O$ requires: N, 7·80; P, 5·75%); UV absorption in water (pH 7): λ_{max} 261 (log ε 3·99), λ_{min} 230 mμ (log ε 3·62); R_f 0·33 (system A), 0·68 (system B). Electrophoretic mobility (pH 8): ca. 0·8 that of uridine 3'-phosphate.

2'-O-Tetrahydropyranyl-5'-O-pivaloyladenosine 3'-phosphate (Xb, R = tBuCO, R' = H)

Compound IXb[1] (0·20 g, 0·46 mmole), pyridinium β-cyanoethyl phosphate[8] (0·93 mmole, from 0·30 g Ba salt) and DCC (0·95 g, 4·6 mmole) were allowed to react together in pyridine (5 ml) soln as above for 3 days at 20°. The reaction mixture was worked up in the manner described above for the corresponding uridine derivative, and the products examined by paper electrophoresis (pH 8) which revealed a principal UV-absorbing component with a mobility ca. 0·3 that of adenosine 3'-phosphate.

A soln of the products was applied to a DEAE-cellulose column (40 cm × 2 cm^2) which was first washed as above, and then eluted with triethylammonium bicarbonate buffer (pH 7·5, linear gradient from 0–0·05M over 2 l.). 25 Ml fractions were collected: fractions 27–35 (average buffer concentration ca. 0·02M) contained the desired β-cyanoethyl ester of 2'-O-tetrahydropyranyl-5'-O-pivaloyladenosine 3'-phosphate (ca. 5000 O.D. units, 75%); fractions 50–57 (average buffer concentration ca. 0·035M) contained a material (360 O.D. units) with an electrophoretic mobility (pH 8) ca. 0·75 that of adenosine 3'-phosphate.

Fractions 27–35 were combined, concentrated to ca. 20 ml, and treated with an excess (with respect to Et_3NH^+) of Dowex-50 (pyridinium form) resin. The latter was removed by filtration, washed with water (10 ml), and the combined filtrate and washings concentrated to ca. 5 ml. Aqueous NH_3 (3N, 18 ml) was added and the soln heated at 50° for 30 min. Air was then bubbled through the cooled soln until the NH_3

* O.D. units at 260 mμ are given, unless otherwise stated.

† Pyridine was added at intervals during this and similar evaporations to prevent the pH from falling below 7

had been removed. Paper electrophoresis (pH 7·5) of the products revealed a principal constituent with a mobility ca. 0·75 that of adenosine 3'-phosphate. Paper chromatography (system A) showed the presence of a main component (R_f 0·58, ca. 90%) and two minor components (R_f's 0·35, 0·80; each ca. 5%). The required 2'-O-tetrahydropyranyl-5'-O-pivaloyladenosine 3'-phosphate was precipitated, in the manner described above, first as a pyridinium salt and then as an almost colourless ammonium salt. Examination of the latter salt (yield 0·15 g) by paper chromatography (system A) showed it to contain ca. 95% of the principal component (R_f 0·58), contaminated only with the less mobile impurity (R_f 0·35).

Acylation of partially de-pivaloylated 2'-O-*tetrahydropyranyl*-5'-O-*pivaloyladenosine* 3'-*phosphate*

The pyridinium salt of the partially de-acylated material was allowed to react with a slight excess of pivaloyl chloride in anhyd pyridine soln for 16 hr at 20°. The products were then treated with MeOH and, after a further 2 hr with a large excess of water. The pH was adjusted to 10 *exactly* by the addition of N NaOH and, after 1½ hr at 20°, lowered to 7 by the addition of Dowex-50 (pyridinium form) resin. Paper chromatography (system A) revealed 2'-O-tetrahydropyranyl-5'-O-pivaloyladenosine 3'-phosphate (R_f 0·58) and a trace contaminant (R_f 0·77) as the sole UV-absorbing products. The major component was isolated as above.

Uridylyl-(5' → 3')-*uridine* 5'-*phosphate* [*pUpU*] (VI; B = B' = *uracil*-1)

Compound VIIa[1] (B = B' = uracil-1) [pyridinium salt from 5300 O.D. units of NH_4^+ salt, 0·265 mmole (based on ε_{260} = 20,000)] was dried by evaporation from anhyd pyridine (4 × 10 ml) soln and then redissolved in pyridine (5 ml). To this soln was added dry pyridinium β-cyanoethyl phosphate[8] (0·54 mmole, from 0·175 g Ba salt) in pyridine (5 ml) soln, DCC (1·1 g, 5·4 mmoles), and anhyd Dowex-50 (pyridinium form, 0·05 g) resin.[1,5] The reaction mixture was sealed and stirred at 20°. After 3 days, water (10 ml) was added and the products stirred at 20° for a further 16 hr before being filtered. The filtrate was extracted with pet. ether (b.p. 40–60°, 2 × 10 ml). The combined aqueous layers were concentrated under reduced press (bath temp < 30°) to ca. ½ volume, treated with 9N aqueous NH_3 (10 ml), and the resulting soln maintained at 50° for 90 min. Paper electrophoresis (pH 8) revealed a principal UV-absorbing component (ca. 90%) with a mobility 0·9 that of uridine 5'-phosphate, and two trace components (each ca. 5%) with mobilities 1·3 and 0·32 that of uridine 5'-phosphate. The latter corresponded to unchanged starting material. Paper chromatography (system C) revealed a major component (R_f 0·47), contaminated with traces of starting material (R_f 0·83) and an unidentified product (R_f 0·08).

The soln was cooled to 20°, and air bubbled through it until most of the ammonia had been removed; it was then concentrated under reduced press (bath temp < 30°) to ca. 5 ml and applied to a column (15 cm × 1·5 cm^2) of Dowex-1 × 2 (Cl^- form) anion-exchange resin. The latter was first washed with water (400 ml) and then eluted with aqueous LiCl–HCl (linear gradient from 0·005M LiCl + 0·005N HCl to 0·15M LiCl + 0·01N HCl over 2 l). 25Ml fractions were collected: fractions 25–35 contained uridylyl-(3' → 5')-uridine* (330 O.D. units); fractions 56–80 contained the required dinucleotide [4250 O.D. units, 80% (based on ε_{260} = 20,000)].

Fractions 56–80 were combined, neutralized (to pH 7) with N LiOH and concentrated (to ca. 5 ml) under reduced press below 30°. The Li salt of VI; (B = B' = uracil-1) was precipitated as a colourless solid by the addition of Me_2CO–EtOH (3:1, v/v; 50 ml), and collected by centrifugation. The product was washed with EtOH (3 × 15 ml) and ether (15 ml), and then dried *in vacuo* over P_2O_5 at 20°, yield 0·17 g; R_f 0·12 (system A), 0·17 (system C). Paper electrophoretic mobility (pH 8): 1·03 that of uridine 5'-phosphate.

When pUpU (1 mg) was treated with pancreatic ribonuclease[16] for 1 hr at 37°, it underwent quantitative conversion to two products, one of which (R_f 0·53; system C) corresponded to uridine. The other product (R_f 0·23; system C) had an electrophoretic mobility (pH 8) 1·13 times that of uridine 5'-pyrophosphate (R_f 0·23; system C).

Cytidylyl-(5' → 3')-*adenosine* 5'-*phosphate* [pApC] (VI; B = *adenine*-9, B' = *cytosine*-1)

Compound VIIa[1] (B = adenine-9, B' = cytosine-1) [pyridinium salt from 2000 O.D. units (at 265 mμ) of NH_4^+ salt, 0·083 mmole, (based on ε_{265} = 24,000)] was evaporated with pyridine (4 × 10 ml), then dissolved in pyridine (5 ml) and treated with Ac_2O (0·2 ml) at 20°. After 16 hr, MeOH (10 ml) was added and, after a further 4 hr, the products were concentrated (to small volume) under reduced press below 30°. 10%

* As this separation took ca. 24 hr and the pH was in the region 2·3–2·0, both the methoxymethylidene and tetrahydropyranyl protecting groups were removed.[1]

Aqueous pyridine (10 ml) was added and after the resulting soln had been allowed to stand at 5° for 16 hr, it was concentrated and re-evaporated several times with pyridine. A soln of the residue in pyridine (3 ml) was added dropwise to ether (30 ml) and the ppt collected, washed and dried as above (in the preparation of 2'-O-tetrahydropyranyl-5'-O-acetyluridine 3'-phosphate); yield of acetylated material, 0·105 g. R_f (system B) 0·90.

The above product was dissolved in N NaOH (2 ml) and the soln allowed to stand at 20° for 1 min *exactly*,[10] after which time an excess of Dowex-50 (pyridinium form) resin was added. Both the soln and resin were applied to a column (8 cm × 1 cm²) of Dowex-50 (pyridinium form) resin, which was drained and washed with 10% aqueous pyridine (6 bed volumes). The total eluate, which was concentrated and re-evaporated several times with pyridine as above, contained largely one UV-absorbing component [R_f (system B) 0·84; UV absorption in water (pH 7): λ_{max} 253, λ_{min} 272, 300 mμ.

To a pyridine (2·5 ml) soln of this material was added pyridinium β-cyanoethyl phosphate[9] (0·166 mmole, from 0·05 g Ba salt), DCC (0·342 g, 1·66 mmole) and anhyd Dowex-50 resin[15] (pyridinium form, 0·05 g). The sealed reactants were stirred for 3 days at 20°, and then worked up (including ammoniacal treatment) in the manner described above (see prep of pUpU). Paper electrophoresis (pH 8) revealed a principal UV-absorbing component with the mobility expected for the partially-protected dinucleotide, contaminated with traces both of starting material and of a more mobile component.

The products were applied to a Dowex-1 × 2 (Cl^- form) anion-exchange column (10 cm × 1·5 cm²) which was eluted with LiCl–HCl (linear gradient from 0·05M LiCl + 0·005N HCl to 0·15M LiCl + 0·01N HCl over 1 l). Fractions (25 ml) 9–15 contained the required dinucleotide [1200 O.D. units at 265 mμ, ca. 60%, (based on ε_{260} = 24,000)]. These fractions were combined and neutralized (to pH 7) with N LiOH. The desired Li salt of cytidylyl-(5' → 3')-adenosine 5'-phosphate was isolated as a colourless solid by precipitation, in the manner described for pUpU; yield, 0·062 g; R_f: 0·04 (system B), 0·45 (system C). Paper electrophoretic (pH 8) mobility: 1·08 that of adenosine 5'-phosphate. UV absorption in water (pH 2·5): λ_{max} 266, λ_{min} 237 mμ.

This material (VI; B = adenine-9, B' = cytosine-1) was unaffected by treatment with pancreatic ribonuclease.[10] However in the presence of *Crotalus adamanteus* snake venom phosphodiesterase, it was completely degraded to adenosine 5'- and cytidine 5'-phosphates [R_f's (system C): 0·52 and 0·40, respectively]. This was confirmed by paper electrophoresis (pH 8).

Cytidylyl-(5' → 3')-uridine 5'-phosphate [pUpC] (VI; B = *uracil*-1, B' = *cytosine*-1)

A soln of VIIa (B = uracil-1, B' = cytosine-1) [pyridinium salt from 300 O.D. units (at 265 mμ) of NH_4^+ salt, 0·015 mmole (based on ε_{260} = 19,000)] in pyridine (3 ml) was treated with Ac_2O (0·03 ml) at 20°, and the products worked up as described in the preparation of pApC. The acetylated material (0·02 g) was obtained as a pale yellow powder following the dropwise addition of its anhyd pyridine (1 ml) soln to ether (15 ml). This material was dissolved in N-NaOH (0·25 ml) and allowed to stand at 20° for 30 sec *only*[10] before the addition of an excess of Dowex-50 (pyridinium form) resin. The resin was removed by filtration and washed with 20% aqueous pyridine (3 × 5 ml). The combined filtrate and washings were concentrated under reduced press below 30°, and finally lyophilized to yield a nearly colourless powder, R_f (system B) 0·80. UV absorption in water (pH 7): λ_{max} 253, 300 mμ.

The latter material was dried by evaporation from anhyd pyridine (4 × 5 ml) soln, dissolved in pyridine (1·5 ml) and treated with an anhyd pyridine soln of pyridinium β-cyanoethyl phosphate[9] (0·031 mmole, from 0·01 g Ba salt), DCC (0·068 g, 0·32 mmole) and anhyd Dowex-50 (pyridinium form, 0·025 g) resin in a sealed vessel at 20° for 3 days. The products were worked up (including ammoniacal treatment) in the manner described above (see preparation of pUpU). Paper electrophoresis (pH 8) revealed a principal UV-absorbing component with 0·95 the mobility of cytidine 5'-phosphate, and two minor components with relative mobilities (also with respect to cytidine 5'-phosphate) of 0·33 and 1·25. Paper chromatography (system B) revealed a principal component (R_f 0·37; ca. 80%) and two minor components (R_f's 0·08, 0·70; each ca. 10%).

A soln of the products was applied to a Dowex 1 × 2 (Cl^- form) anion-exchange column (3 cm × 1·2 cm²) which was washed thoroughly with water and then eluted in turn with 500 ml each of (a) 0·001 N-, (b) 0·005 N- and (c) 0·01 N-HCl. The eluate from (b) contained UpC (45 O.D. units at 265 mμ), and the eluate from (c) contained the desired VI (B = uracil-1, B' = cytosine-1) [170 O.D. units, at 265 mμ, ca. 60% (based on ε_{260} = 19,000)]. The latter material was isolated by the precipitation technique described above, as a colourless Li salt; yield 0·01 g. R_f 0·14 (system A), 0·21 (system C). Electrophoretic mobility (pH 8):

0·97 that of uridine 5'-phosphate; 0·96 that of pUpU. UV absorption in water: at pH 2, λ_{max} 270, λ_{min} 237 mμ; at pH 7, λ_{max} 266, λ_{min} 235 mμ.

pUpC was quantitatively converted by pancreatic ribonuclease[16] into two products, one of which [R_f 0·66 (system C)] corresponded to cytidine. The other product [R_f 0·24 (system C)] was identical to the nucleotidic product obtained by digesting pUpU with pancreatic ribonuclease (see above).

2'-O-Tetrahydropyranyluridylyl-(3' → 5')-2-O-tetrahydropyranyluridylyl-(3' → 5')-2',3'-O-methoxymethylideneuridine (XIa)

The pyridinium salts of VIIa[1] (B = B' = uracil-1) [from 1500 O.D. units of NH_4^+ salt, 0·075 mmole (based on ε_{260} = 20,000)] and Xa (R = Ac, R' = H) [from 0·04 g of NH_4^+ salt, 0·075 mmole] were dried by evaporation from anhyd pyridine (4 × 10 ml) soln, and dissolved in DMF (2 ml) and pyridine (6 ml). DCC (0·155 g, 0·75 mmole) and anhyd Dowex-50 (pyridinium form, 0·05 g)[15] were added, and the sealed reaction mixture stirred at 20°. After 24 hr, a further quantity of the mononucleotide (from 0·02 g of NH_4^+ salt, 0·038 mmole) was added and the reaction allowed to continue. After a further 48 hr, water (10 ml) was added and the reaction mixture stirred at 20° for 6 hr. The products were then extracted with pet. ether (b.p. 40–60°, 10 ml), the aqueous layer filtered, treated with aqueous tetraethylammonium hydroxide (1·7N, 0·05 ml) and concentrated (to ca. 2 ml) under reduced press below 30°. Aqueous methanolic (1:1, v/v) tetraethylammonium hydroxide (2 ml) of 0·8M, ca. 1·5 mmoles) was added and the soln allowed to stand at 20° for 16 hr before it was neutralized by the addition of an excess of Dowex-50 (pyridinium form) resin. The resin was removed by filtration, washed with water, and the filtrate and washings combined. Paper chromatography (system B) revealed a principal UV-absorbing component* (R_f 0·74), and two minor components (R_f's 0·55, 0·86).

The soln of products was applied to a column (68 cm × 4·5 cm^2) of DEAE-cellulose (HCO_3^- form), which was washed with 0·002M triethylammonium bicarbonate buffer (pH 7·5) until the eluate no longer absorbed in the UV. The column was then eluted with triethylammonium bicarbonate buffer (pH 7·5, linear gradient from 0·002–0·2M over 2 l). 25 Ml fractions were collected: fractions 20–31 (see below) [average buffer concentration ca. 0·06M] contained a material (1710 O.D. units) with electrophoretic mobility (pH 8) 0·33 that of uridine 5'-phosphate; fractions 33–41 (average buffer concentration ca. 0·09M) contained mainly 2'-O-tetrahydropyranyluridine 3'-phosphate (290 O.D. units; paper electrophoretic mobility 0·93 that of uridine 5'-phosphate); fractions 42–53 (average buffer concentration ca. 0·12M) contained the desired partially-protected UpUpU XIa [1980 O.D. units, yield 57% (based on ε_{260} = 30,000), electrophoretic mobility (pH 8) 0·55 that of uridine 5'-phosphate]; fractions 54–59 (average buffer concentration 0·14M, 140 O.D. units) contained the partially-protected UpUpU, contaminated with an electrophoretically (pH 8) more mobile product.

Fractions 42–53 were concentrated just to dryness (under reduced press below 30°), redissolved in 0·01M aqueous NH_3 (5 ml) and lyophilized. The ammonium salt of the partially-protected UpUpU (XIa) (0·064 g) was isolated as a colourless ppt by adding an anhyd soln of it in pyridine to ether (see above). UV absorption in water (pH 7): λ_{max} 262 mμ. R_f: 0·37† (system A), 0·68† (system B). Electrophoretic mobility (pH 8): 0·93 that of UpUpU, 0·55 that of uridine 5'-phosphate.

Fractions 20–31 were similarly concentrated, treated with aqueous NH_3 and lyophilized. Some of this product was dissolved in 60% AcOH and the soln allowed to stand at 20° for 6 hr. Paper chromatography (systems A and B) and electrophoresis (pH 8) of the hydrolysate revealed approximately equal quantities of two components which corresponded[17] to Up and UpU.

Uridylyl-(3' → 5')-uridylyl-(3' → 5')-uridine [UpUpU] (XIIa)

(a) An aqueous soln of the above partially-protected UpUpU (XIa) (180 O.D. units) was adjusted to pH 8 and applied to a column (5·5 cm × 3 cm^2) of Dowex-1 × 2 (Cl^- form) anion-exchange resin, which was washed with water (500 ml) and then successively eluted (within ca. 20 hr) with: (a) 0·01N HCl (500 ml), (b) 0·01N HCl + 0·01M LiCl (500 ml) and (c) 0·01N HCl + 0·1M LiCl (1000 ml). 25 Ml fractions were collected; those fractions containing UV-absorbing material eluted with soln (c) were combined (150 O.D. units at 262 mμ), adjusted to pH 7 with N LiOH, and then concentrated (under reduced press

* This material is probably a mixture of the desired partially-protected UpUpU and starting material (i.e. partially-protected UpU). The latter has R_f (system B) 0·74.

† A slight impurity (not > 5%) with R_f 0·48 (system A), 0·78 (system B) was detected in this material.

below 30°) to a ca. 5 ml. A soln of $CaCl_2$ (0·03 g) in EtOH (2 ml) was added, followed by (2:1, v/v) EtOH–Me_2CO (75 ml). The calcium salt of UpUpU, obtained as a colourless precipitate, was washed with EtOH–Me_2CO, EtOH, and finally ether. R_f (system C): 0·11. Electrophoretic mobility (pH 8): 0·55 that of uridine 3'-phosphate. UV absorption in water (pH 7): λ_{max} 262 mμ.

(b) A soln (ca. pH 2) of the partially-protected UpUpU (XIa) (ca. 6 mg) in 0·01N HCl (5 ml) was allowed to stand at 20° for 10 hr, and was then neutralized (to pH 7) with dil aqueous NH_3 before lyophilization. Paper chromatography (system C) revealed a principal UV-absorbing component (R_f 0·11, ca. 95%), contaminated with a trace of an impurity (R_f 0·30).

One-half of this material was treated with pancreatic ribonuclease, under the usual conditions.[16] Paper chromatography (system C) of the digest revealed two products (R_f's 0·30, 0·48) and no undegraded UpUpU (R_f 0·11). Paper electrophoresis (pH 8) resolved the digest into two components with the mobilities of uridine and uridine 3'-phosphate.

2'-O-*Tetrahydropyranyluridylyl*-(3' → 5')-2'-O-*tetrahydropyranyladenylyl*-(3' → 5')-2',3'-O-*methoxymethylideneuridine* (XIb).

The pyridinium salts of VIIa[1] (B = adenine-9, B' = uracil-1) [from 1500 O.D. units of triethylammonium salt (0·058 g), ca. 0·06 mmole (based on ε_{260} = 25,000)] and Xa (R = Ac, R' = H) [from 0·035 g of NH_4^+ salt, 0·063 mmole], DCC (0·13 g, 0·63 mmole), anhyd Dowex-50 (pyridinium form, 0·05 g) resin,[15] pyridine (6 ml) and DMF (2 ml) were stirred together in a sealed flask at 20°. After 40 hr, more of the dinucleoside phosphate component (from 0·018 g of triethylammonium salt, 0·02 mmole) was added, the flask re-sealed and the reaction allowed to continue for a further 32 hr. The products were then worked up in the manner described in the preparation of partially-protected UpUpU (see above). Paper electrophoresis (pH 8) of the de-acylated products showed two main UV-absorbing components with mobilities of 0·22 (corresponding to partially-protected ApU), and 0·43 that of uridine 3'-phosphate.

A soln of this material was applied to a column (56 cm × 3·5 cm^2) of DEAE-cellulose (HCO_3 form), which was washed with 0·002M triethylammonium bicarbonate buffer (pH 7·5) until the eluate no longer absorbed in the UV. The column was then eluted with triethylammonium bicarbonate buffer (pH 7·5, linear gradient from 0·002–0·2M over 2 l). 25 Ml fractions were collected: fractions 31–35 (average buffer concentration ca. 0·08M) contained partially-protected ApU VIIa (B = adenine-9, B' = uracil-1); fractions 54–60 (average buffer concentration ca. 0·14M) contained the desired partially-protected UpApU (XIb) [1200 O.D. units, 54% (based on ε_{260} = 35,000)]. Small quantities of unidentified UV-absorbing materials were eluted before each of the main components.

Fractions 54–60 were combined, concentrated (under reduced press. below 30°) just to dryness, redissolved in water and lyophilized to give XIb as a colourless powder. UV absorption in water (pH 7): λ_{max} 262, λ_{min} 233 mμ. R_f (system A) 0·40. Electrophoretic mobility (pH 8): 0·45 that of uridine 3'-phosphate.‡

Uridylyl-(3' → 5')-*adenylyl*-(3' → 5')-*uridine* [UpApU] (XIIb)

A soln of the above partially-protected UpApU (XIb) (ca. 20 O.D. units) in 0·01N HCl (0·5 ml) was allowed to stand at 20° for 8 hr. Aqueous NH_3 was added until the pH increased to 7·5, and the soln then lyophilized. The ammonium salt of UpApU (XIIb), so obtained, was paper chromatographically homogeneous in systems B and C (R_f 0·18 and 0·31, respectively).

A portion of XIIb was treated with pancreatic ribonuclease,[16] and completely digested to two components [R_f's (system C) 0·35, 0·48] corresponding to Up and ApU, respectively. This was confirmed by paper electrophoresis (pH 8). XIIb was completely degraded in the presence of *Crotalus adamanteus* snake venom phosphodiesterase to approximately equimolecular amounts of uridine 5'-phosphate [R_f: 0·23 (system B), 0·30 (system C)], adenosine 5'-phosphate [R_f: 0·17 (system B), 0·57 (system C)] and uridine [R_f: 0·81 (system B), 0·54 (system C)]. Paper electrophoresis (0·2M sodium borate, pH 9) of the digest revealed 3 anionic components, with relative mobilities 1·0, 0·84 and 0·44, corresponding to uridine 5'-phosphate, adenosine 5'-phosphate and uridine, respectively.

2'-O-*Tetrahydropyranyladenylyl*-(3' → 5')-2'-O-*tetrahydropyranyladenylyl*-(3' → 5')-2',3'-O-*methoxymethylideneuridine* (XIc)

The pyrdinium salts of VIIa[1] (B = adenine-9, B' = uracil-1) [from 0·035 g (900 O.D. units) of triethylammonium salt, 0·036 mmole (based on ε_{260} = 25,000)] and Xb (R = tBuCO, R' = H) [from 0·04 g

‡ A trace (1·2%) impurity, with a mobility 0·54 that of uridine 3'-phosphate, was detected.

(700 O.D. units) of NH_4^+ salt, 0·046 mmole (based on ε_{260} = 15,000)], DCC (0·085 g, 0·4 mmole), anhyd Dowex-50 (pyridinium form, ca. 0·025 g) resin,[15] pyridine (3 ml) and DMF (1 ml) were stirred together in a sealed flask at 20°. After 24 hr, more of the above dinucleoside phosphate component (from 0·017 g of triethylammonium salt, 0·02 mmole) was added, the flask re-sealed and the reaction allowed to continue for a further 48 hr. Water (5 ml) was added and the reaction mixture stirred at 20° for 10 hr. The products were then extracted with pet. ether (b.p. 40–60°, 2 × 5 ml), the aqueous layer filtered, treated with aqueous tetraethylammonium hydroxide (1·7N, 0·04 ml) and concentrated (to ca. 1 ml) under reduced press. below 30°. Aqueous methanolic (1:1, v/v) tetraethylammonium hydroxide (0·2 ml of 0·8M) was added, and the soln allowed to stand at 20° for 16 hr before it was neutralized by the addition of an excess of Dowex-50 (pyridinium form) resin. The resin was removed by filtration, washed with water, and the filtrate and washings combined. Paper electrophoresis (pH 8) showed a major UV-absorbing component with a mobility 0·44 that of adenosine 3'-phosphate, some unchanged partially-protected ApU, and a more mobile trace component.

The soln of products was applied to a column (50 cm × 3 cm²) of DEAE-cellulose (HCO_3^- form), which was washed with 0·002M triethylammonium bicarbonate buffer (pH 7·5) until the eluate no longer absorbed in the UV. The column was then eluted with triethylammonium bicarbonate buffer (pH 7·5, linear gradient from 0·002–0·2M over 2 l.). 25 Ml fractions were collected: fractions 23–29 (average buffer concentration ca. 0·06M) contained unchanged partially-protected ApU (200 O.D. units at 260 mμ); fractions 58–64 (average buffer concentration ca. 0·15M) contained the desired partially-protected ApApU (XIc) [855 O.D. units, 54% (based on ε_{260} = 40,000)]

The latter fractions were evaporated (under reduced press. below 30°) *just* to dryness, redissolved in water and then lyophilized to give a colourless powder. Paper electrophoresis (pH 8) showed a main UV-absorbing component (ca. 95%) with mobility 0·40 that of adenosine 3'-phosphate, contaminated with a more mobile material (ca. 5%). R_f of partially-protected ApApU: 0·75 (system A), 0·47 (system B).

Adenylyl-(3' → 5')-adenylyl-(3' → 5')-uridine [ApApU] (XIIc)

Partially-protected ApApU (XIc) (ca. 420 O.D. units) was dissolved in water (ca. 5 ml) and the pH adjusted to 2 (pH meter) by the addition of dil HCl. After it had stood at 20° for 6 hr, the soln was neutralized (to pH 7·5) with dil aqueous NH_3 and lyophilized.

A soln of this material was applied to a column (40 cm × 3 cm²) of DEAE-cellulose (HCO_3^- form), which was eluted with triethylammonium bicarbonate buffer (pH 7·5, linear gradient from 0·0–0·2M over 2 l.). 25 Ml fractions were collected: fractions 52–56 (average buffer concentration ca. 0·14M) and fractions 60–66 (average buffer concentration ca. 0·16M) contained UV-absorbing material. Fractions 60–66, which contained most of the UV-absorbing material, were combined and lyophilized to give ApApU (XIIc) as a colourless solid. R_f: 0·14 (system A), 0·40 (system C); electrophoretic mobility (pH 8) 0·50 that of adenosine 5'-phosphate. UV absorption in water (pH 7): λ_{max} 260, λ_{min} 233 mμ.

A portion of XIIc was completely degraded in the presence of *Crotalus adamanteus* snake venom phosphodiesterase to approximately equimolar amounts of uridine 5'-phosphate [R_f (system C) 0·30], adenosine 5'-phosphate [R_f (system C) 0·57], and adenosine [R_f (system C) 0·88]. Paper electrophoresis (0·1M NaOAc buffer, pH 4·5) revealed a cationic component corresponding to adenosine, and two anionic components corresponding to adenosine 5'- and uridine 5'-phosphates.

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