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THE SEPARATION OF 2'-5' DINUCLEOSIDE MONOPHOSPHATES FROM THE CORRESPONDING 3'-5' ISOMERS ON A DEAE-SEPHADEX A-25 COLUMN

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SUMMARY

The separation of 2'-5' dinucleoside monophosphates from their corresponding 3'-5' isomers on a DEAE-Sephadex A-25 (bicarbonate form) column is described. The column is loaded with a mixture of 2'(3')-5' dinucleoside monophosphate and eluted with a linear gradient of ammonium bicarbonate (from 0.02 to 0.15 *M*). The two isomers come out in two different peaks, and the nucleotidic material is isolated by lyophylization.

INTRODUCTION

Dinucleoside monophosphates* represent the smallest diester units of ribonucleic acids. The dinucleoside monophosphates are used therefore for studying the specificity of different RNases as well as for conformational studies. Recently UKITA *et al.*[1] reported on the synthesis of dinucleoside monophosphates containing a mixture of 2'-5' and 3'-5' linkages. The two isomers were separated from each other by chromatography on a Dowex 1 X2 (formate form) column. The elution was carried out with formic acid, and special care had to be taken in order to avoid phosphoryl migration.

In the present communication the separation of 2'-5' dinucleoside monophosphates from the corresponding 3'-5' isomers on DEAE-Sephadex A-25 column (bicarbonate form) is described. The elution is carried out with a linear gradient of ammonium bicarbonate (from 0.02 to 0.15 *M*) and the nucleotidic material is isolated by lyophylization.

EXPERIMENTAL

Materials

DEAE-Sephadex A-25 with a capacity of 3.5 mequiv./g was purchased from Pharmacia, Uppsala, Sweden.

Reagent grade pyridine was distilled and dried over calcium hydride. All evaporations were carried out using a rotary evaporator under reduced pressure.

* Abbreviations used: A, adenosine; C, cytidine; G, guanosine; U, uridine. ApN, CpN, GpN, UpN, NpG, dinucleoside monophosphate where N stands for A, C, G or U.

Pancreatic ribonuclease (2×crystallized) and ribonuclease T_1 were purchased from Calbiochem, Calif., U.S.A. Ribonuclease T_2 was prepared according to the procedure described by UCHIDA AND EGAMI[2].

Paper electrophoresis (0.03 *M* phosphate buffer pH 7.1) was performed in a high-voltage apparatus (4,500 V; 45 V/cm) in which paper was immersed in a high boiling petroleum fraction (varsol).

The column chromatography apparatus consisted of a 3.2×55 cm column, a UV flow-through cell, 254 nm (LKB Uvicord 1), recorder (LKB 6520) and an LKB fraction collector.

Protected nucleosides

2',3'-Dibenzoyluridine, N,2',3'-tribenzoyladenosine and N,2',3'-tribenzoylcytidine were prepared according to LOHRMANN AND KHORANA[3]. 2',3'-Methoxymethylidene guanosine was prepared according to GRIFFIN *et al.*[4].

Acetylation of nucleoside 2'(3')-phosphate. General method

Nucleoside-2'(3')-phosphate (10 mmoles) and tetraethylammonium acetate (50 mmoles) were rendered anhydrous by repeated evaporations of dry pyridine. During the last evaporation the suction under vacuum was prolonged to obtain a gum free of pyridine. Acetic anhydride (150 mmoles) was added and the sealed reaction mixture was kept at room temperature for 3 days. A mixture of methanol–pyridine (4:1) (120 ml) was added to destroy excess acetic anhydride, and after 30 min at room temperature the solution was evaporated to dryness. The residue was dissolved in 2 % aqueous pyridine (30 ml) and the solution was passed through a column of Dowex 50 X8 (pyridinium form) ion-exchange resin (300 ml of wet resin). The column was eluted with 2 % aqueous pyridine. To the total effluent (about 500 ml) pyridine (50 ml) was added and the solution was kept at room temperature until no acetyl phosphate derivative could be detected by high voltage paper electrophoresis at pH 7.1. The solution was then concentrated to a small volume under reduced pressure, and rendered anhydrous by co-evaporation with dry pyridine. Finally, the residue was dissolved in dry pyridine (30 ml) and then added slowly under stirring to an excess of anhydrous ether (500 ml). The fine precipitate which resulted was collected by filtration through a glass filter, washed with dry ether and dried in a vacuum desiccator over phosphorous pentoxide. The yield of the dry powder was 95%.

Synthesis of 2'(3')-5' dinucleoside monophosphate. General method

A mixture of N,3'(2')5'-triacetylnucleoside-2'(3')-phosphate (1 mmole) and the protected nucleoside (2 mmoles) was rendered anhydrous by repeated evaporation of dry pyridine. Finally, the residue was dissolved in anhydrous pyridine (10 ml) and triisopropylbenzene sulfonyl chloride (TPS) (600 mg) was added. The reaction mixture was kept at room temperature overnight and was then treated with 50 % aqueous pyridine (10 ml). After 30 min at room temperature the solvent was removed under reduced pressure and the protecting groups were removed by treatment with 15 *M* methanolic ammonia for 24 h. (In the case of NpG the residue was treated with 80% acetic acid for 15 h prior to the ammonia treatment.) The solvent was then evaporated under reduced pressure, the residue was dissolved in 0.02 *M*

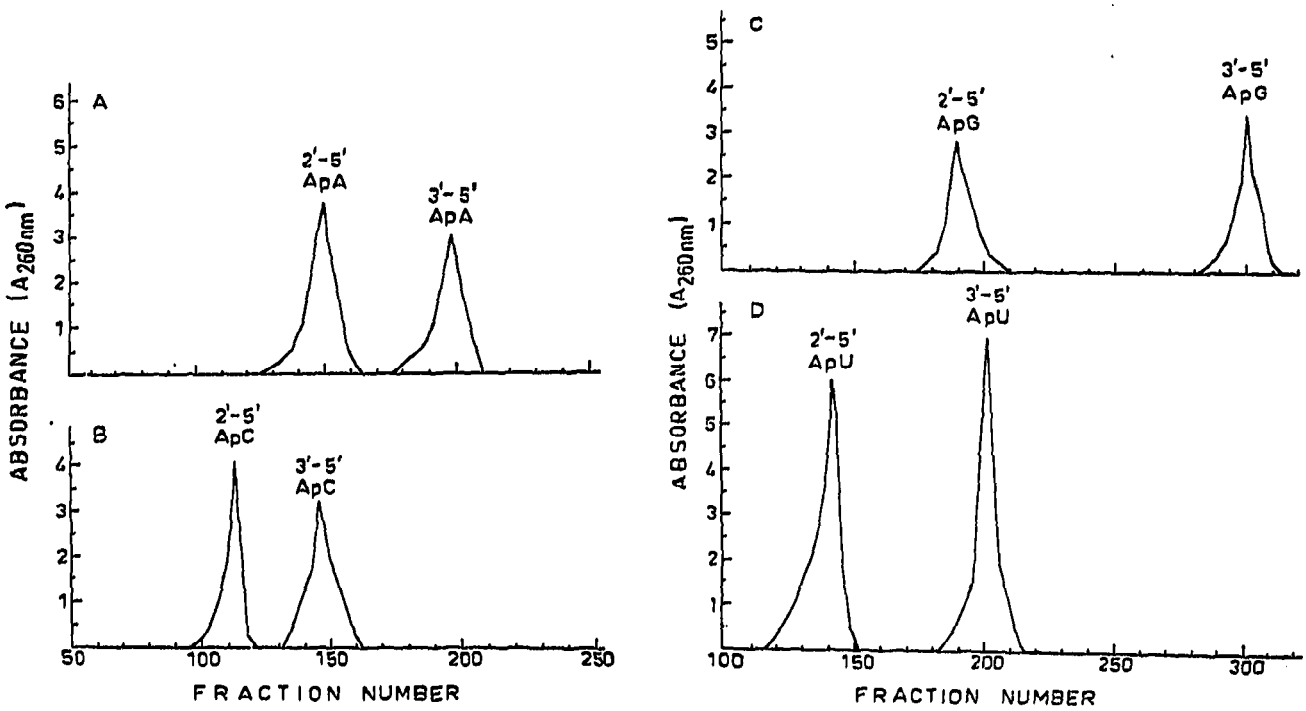


Fig. 1. Chromatography of A2'(3')-5' N on DEAE–Sephadex A-25 (bicarbonate) column. For details see text.

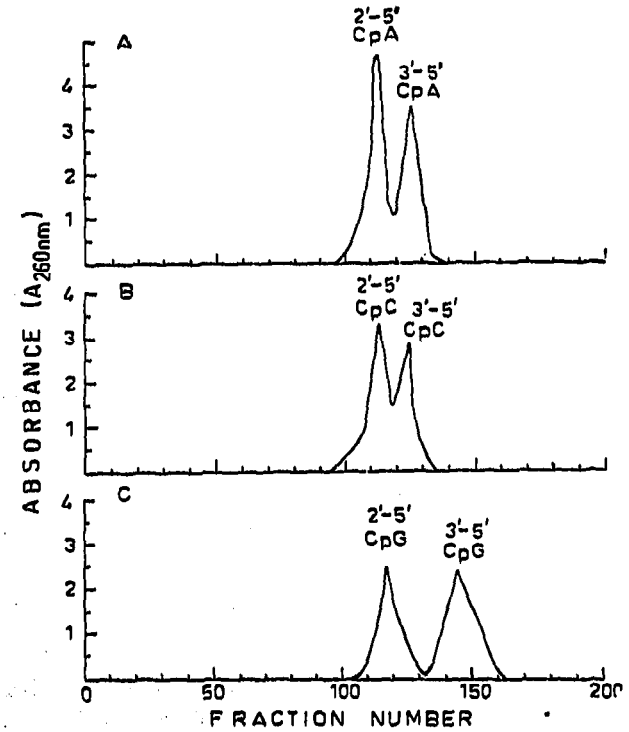


Fig. 2. Chromatography of C2'(3')-5' N on DEAE–Sephadex A-25 (bicarbonate) column. For details see text.

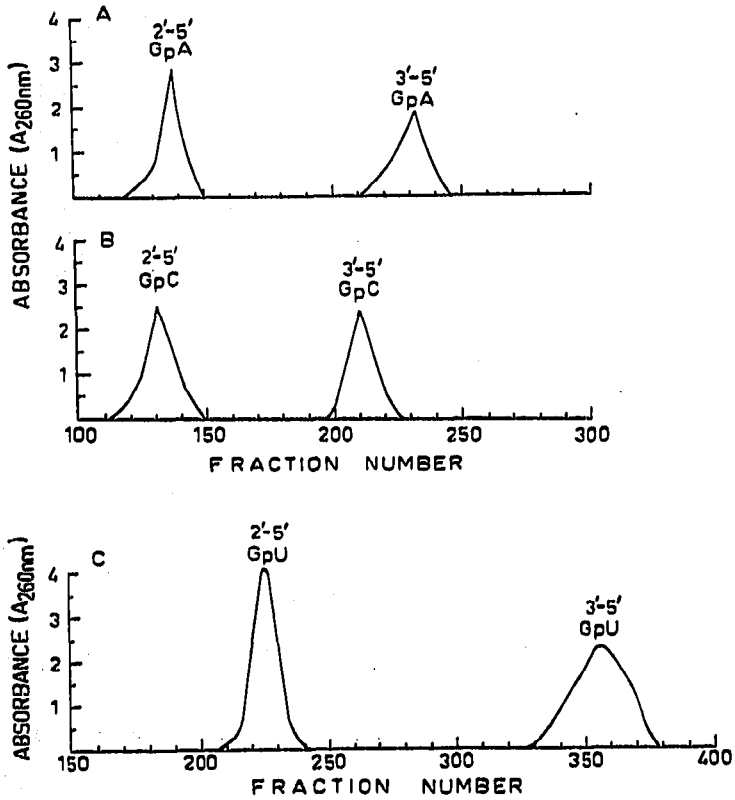


Fig. 3. Chromatography of G2'(3')-5' N on DEAE-Sephadex A-25 (bicarbonate) column. For details see text.

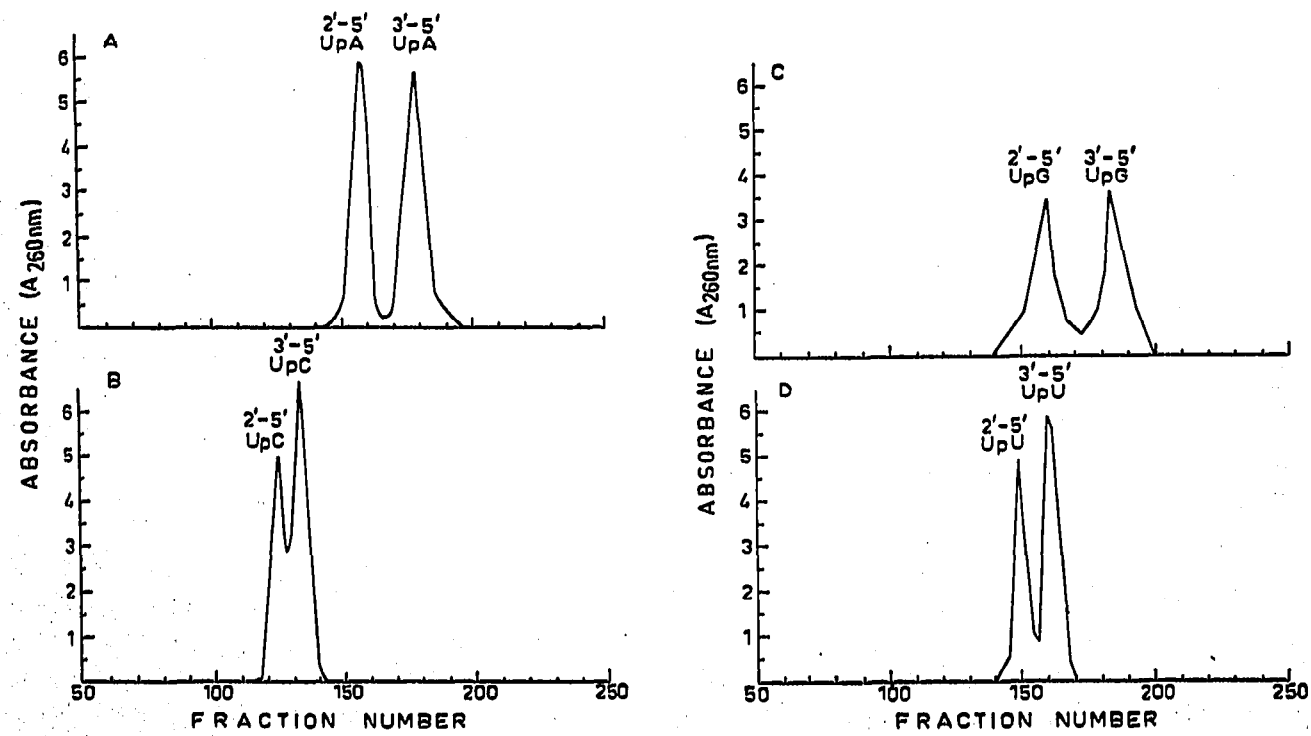
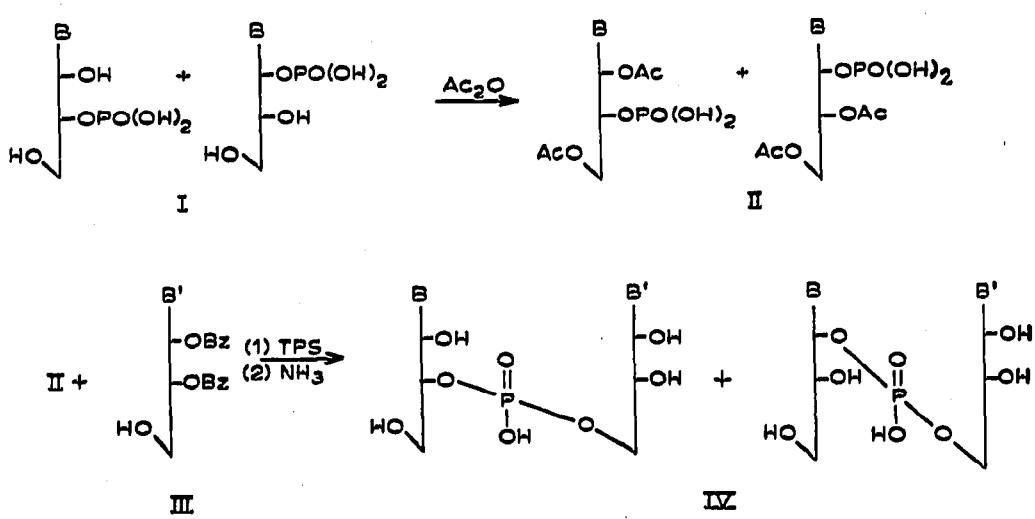


Fig. 4. Chromatography of U2'(3')-5' N on DEAE-Sephadex A-25 (bicarbonate) column. For details see text.

ammonium bicarbonate and the insoluble material was removed by filtration (NpG was dissolved in the presence of 7 *M* urea.) A portion of the solution containing 0.1 mmole of the product was placed on a DEAE–Sephadex A-25 (bicarbonate) column (3.2 × 55 cm). The column was eluted with a linear gradient of ammonium bicarbonate. The mixing vessel contained 4 l of 0.02 *M* ammonium bicarbonate and the reservoir contained an equal volume of 0.15 *M* ammonium bicarbonate. 20-ml fractions were collected at a flow rate of 2.8 ml/min. The different fractions were pooled, evaporated to a small volume under reduced pressure and lyophylized. The elution patterns of the different dinucleoside monophosphates are given in Figs. 1–4.

RESULTS AND DISCUSSION

The synthesis of dinucleoside monophosphate containing a mixture of 2'-5' and 3'-5' linkages (IV) was achieved in analogy to the synthesis of 3'-5' dinucleoside monophosphate described by LOHRMANN AND KHORANA[3]. Thus, the nucleoside-2'(3')-phosphate (I) was acetylated with acetic anhydride in the presence of 5-fold (instead of 10-fold) excess of tetraethylammonium acetate. The acetylated product, N,3'(2',)



B,B', adenine, cytosine, guanosine, uracil; Ac, acetyl; Bz, benzoyl; TPS, triisopropylbenzene sulfonyl chloride.

5'-triacetyl-nucleoside-2'(3')-phosphate (II), was condensed with a 2-fold excess of N,2',3' protected nucleoside (III) in the presence of triisopropylbenzene sulfonyl chloride. The condensation was quantitative based on the amount of nucleotide used.

After removal of all the protecting groups, the reaction mixture was put on a DEAE–Sephadex A-25 (bicarbonate) column and the material was eluted with a linear gradient of ammonium bicarbonate at pH 8.6 (from 0.02 to 0.15 *M*). Figs. 1–4 show the elution pattern of the mixtures containing 2'-5' and 3'-5'dinucleoside monophosphates. The first material eluted from the column was the free nucleoside (not shown) and in most cases there was no free mononucleotide. The different fractions shown in Figs. 1–4 were pooled, concentrated to a small volume by evaporation under reduced pressure and lyophylized. Aliquots of each peak were treated with the appropriate ribonuclease (pancreatic ribonuclease in the case of CpN and

UpN, ribonuclease T_1 in the case of GpN and ribonuclease T_2 in the case of ApN) and the products were analyzed by high-voltage paper electrophoresis. All the dinucleoside monophosphates containing the natural 3'-5' linkage were completely degraded to nucleoside-3'-phosphate and the free nucleoside, while those with the unnatural 2'-5' linkage were resistant to the enzymatic hydrolysis. Treatment of each peak with 0.5 *N* NaOH resulted in the formation of nucleoside-2'(3')-phosphate and the free nucleoside.

Fig. 1 shows the separation of A2'-5'N from the corresponding A3'-5'N. In Fig. 1A, the first peak was resistant to enzymatic hydrolysis (A2'-5'A) while the second peak was degraded completely by ribonuclease T_2 to adenosine-3'-phosphate and adenosine. Similarly, the first peaks in Figs. 1B–1D were resistant to enzymatic hydrolysis (A2'-5'C, A2'-5'G and A2'-5'U, respectively) while the second peak degraded completely to adenosine-3'-phosphate and the appropriate nucleoside.

Figs. 2–4 show the separation of C2'-5'N, G2'-5'N and U2'-5'N from the corresponding C3'-5'N, G3'-5'N and U3'-5'N. CpU was the only dinucleoside monophosphate where no separation could be detected between the 2'-5' and 3'-5' isomers (not shown in the figure). As a rule, each dinucleoside monophosphate containing the 2'-5' linkage was eluted from the DEAE–Sephadex A-25 column before the corresponding dinucleoside monophosphate containing the natural 3'-5' linkage. A similar order of elution was found when mixtures of 2'-5' and 3'-5' dinucleoside monophosphates were chromatographed on a Dowex 1 X2 (formate form) column[1] or on ion-exchange thin layers[5].

The separation of oligonucleotides on DEAE–Sephadex columns depends not only on their chain length (net charge) but also on secondary binding forces such as hydrophobic and hydrogen bonds. These forces depend on the base composition of the oligonucleotide—those containing purines for example are more strongly held on the column than those containing pyrimidines[6]. In the case of 2'-5' and 3'-5' dinucleoside monophosphate, the two isomers have the same chain length and the same base composition and the only difference between them is the nature of the phosphate diester linkage. It is very likely therefore that the different elution patterns of the 2'-5' and the corresponding 3'-5' dinucleoside monophosphates are due to conformational differences. Conformational differences between A2'-5'N and A3'-5'N, for example, were recently suggested by KONDO *et al.*[7]. This suggestion is based on studies of proton magnetic resonance and circular dichroism spectra of the dinucleoside monophosphates containing the 2'-5' and the 3'-5' linkages.

The separation of 2'-5' dinucleoside monophosphates from the corresponding 3'-5' isomers on DEAE–Sephadex A-25 column can be used for analytical as well as for preparative purposes.

The advantage of DEAE–Sephadex A-25 (bicarbonate) column over Dowex 1 X2 (formate) column is the stability of the dinucleoside monophosphates at pH 8.6 (no phosphoryl migration occurs under these conditions) and the ease of the removal of the ammonium bicarbonate from the effluent.

ACKNOWLEDGEMENTS

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NOTE ADDED IN PROOF

Recently it was reported by MIURA AND UEDA[8] on the separation of 2'-5'ApU from 3'-5' ApU on DEAE-cellulose column.

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