THE SYNTHESIS OF NUCLEOSIDE 2'(3')-PHOSPHATE 5'-TRIPHOSPHATES

E. MESSENS and M. VAN MONTAGU
Laboratory of Histology and Genetics, State University of Ghent, Ledeganckstraat 35, Ghent, Belgium

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1. Introduction

Recently it was shown that the RNA chain of the RNA bacteriophages [1—4] and T_4 messenger RNA [5] start at the 5' terminus with a 5'-triphosphate group. This being in contrast to the free 5'-hydroxyl group of TMV RNA [6] or the monophosphorylated 5'-hydroxyl group of all ribosomal [5, 7] and transfer RNA [8] examined so far. After base hydrolysis the 5' terminal nucleotide of the former RNA molecules will be released as ribonucleoside 2'(3')-mono-, 5'-triphosphate (pppNp).

In order to rapidly identify the terminal nucleotide in a hydrolysate of labelled RNA, it is necessary to have the four authentic nucleoside tetraphosphates which have not previously been synthesized. We prepared them in a 75% yield out of the nucleoside 2'(3'),5'-diphosphates (pNp). The different steps are represented in fig. 1.

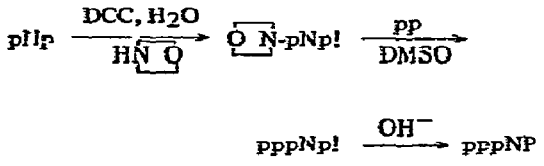

Fig. 1.

Abbreviations used: N = nucleoside, Np = nucleoside 2'(3')-pyrophosphate, pN = nucleoside 5'-phosphate, ppN = nucleoside 5'-pyrophosphate, pppN = nucleoside 5'-triphosphate, Np! = nucleoside 2'3'-cyclic phosphate, O N-pNp! = nucleoside 2'3'-cyclic phosphate 5'-phosphoromorpholidate, ppNp! = nucleoside 2'3'-cyclic phosphate 5'-pyrophosphate and ppNp = 2'(3')-nucleosidephosphate 5'-pyrophosphate.

The syntheses of the nucleoside 2'(3'),5'-diphosphates are rather well documented [9, 10, 12].

We synthesized the purine diphosphates by phosphorylation of nucleoside with β-cyanoethylphosphate [9]. The pyrimidine diphosphates were prepared by phosphorylation with polyphosphoric acid [10]. The final purification of each pNp was carried out on a Dowex-2 column using an ammonium formate gradient 0.1—1.0 N (-1.5 N for pGp) at pH 3.8.

Moffatt and Khorana [11, 12] prepared adenosine 2',3'-cyclic phosphate 5'-phosphoromorpholidate directly from pAp in 95% yield. We proceeded as indicated by these authors and we obtained the corresponding derivatives of all four nucleosides in 95% yield.

The condensation with pyrophosphate is complete in one day at 37° when performed under sufficiently anhydrous conditions and with dimethyl sulfoxide (DMSO) as solvent [13]. Longer treatment results in some ppNp! and pNp!.

The latter compounds and some polyphosphate derivatives, which were not further investigated, are also formed when after the completion of the reaction the mixture is concentrated instead of directly loaded to a DEAE-Sephadex column. The decyclization of the 2',3'-cyclic ester at 55° is complete in 15 min by adding the same volume 0.8 N KOH to the reaction mixture, although treatment up to several hours does not effect any breakdown of the desired compounds. Alternatively the pppNp! may be decyclisized after the column separation but then 3 hr of the same base treatment are required because of the absence of DMSO.

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Table 1

Relative rates of the different nucleotide compounds in solvent A; isobutyric acid: 1 M NH_4OH (5:3, v/v) and B; ethanol: 1 M ammonium acetate pH 5.0 (5:2, v/v).

	N	Np	Np!	pNp	pNp!	O N-pNp!	ppNp	ppNp!	pppNp	pppNp!
40 hr solvent A 100 = Front	U	44	59	34	40	(49)d	23	26	18	19
	C	59	80	39	47	(56)d	28	31	22	23
	A	66	83	41	52	(58)d	32	35	27	28
	G	39	52	23	30	(41)d	18	20	15	16
55 hr solvent B 100 = Gp	U	170	286	57	102	(d)	20	62	11	25
	C	145	261	39	84	(d)	15	45	6	19
	A	124	248	38	83	(d)	15	43	7	19
	G	100	220	24	68	(d)	12	30	5	16

Abbreviations used: d = decomposed U = uridine C = cytidine A = adenosine G = guanosine

2. Experimental

As an example we describe the synthesis of pppGp, the synthesis of the three other tetraphosphates is analogous except where indicated.

A mixture of pyridinium β-cyanoethyl phosphate (10 mmole) and guanosine (1 mmole) was rendered anhydrous by coevaporations with pyridine and redissolved in 10 ml pyridine. DCC (20 mmole) was added and the sealed mixture was shaken at 37° for two days. Pyridine was removed by several coevaporations with water. The residue was heated for two hours in 0.5 N lithium hydroxide (40 ml). The insoluble dicyclohexyl urea (DCU) was removed by filtration. The total filtrate was applied to a 2.6×25 cm column of Dowex 2 (formate). The elution was with a 6 l gradient of 0.2 M–1.5 M ammonium formate at pH 3.8. The diphosphate peak eluted at 0.8 M (yield 75%). It was rendered salt free by several lyophilizations at 40° and 10^{-5} mm Hg (mercury diffusion pump) and was brought in the morpholinium form with Dowex 50. After being dissolved in a mixture of water (9 ml), *t*-butyl alcohol (15 ml) and morpholine (6 mmole), the pNp solution was refluxed and 15 mmole DCC, dissolved in 15 ml *t*-butyl alcohol was added dropwise over a period of 6 hr [12] and refluxing was continued for a further 6 hr. The *t*-butyl alcohol was removed by coevaporation with water. The residue was taken up in 30 ml of water and extracted with petroleum ether, DCU was removed by filtration, and the filtrate was twice more extracted with ether before drying under high vacuum. The bis-(4-morpholine N,N′-dicyclohexylcarboxammidinium salt of guanosine 2′,3′-cyclic phosphate 5′-phosphomorpholidate was rendered anhydrous by coevaporations consecutively with pyridine (five times), benzene (five times), DMSO (twice), and finally dissolved in 30 ml DMSO. To this was added 3.75 mmole (5 eq.) anhydrous pyrophosphate suspended in 7.5 ml DMSO (two mmole tributylamine was added to one mmole pyrophosphoric acid suspended in 75% aqueous pyridine; the mixture was evaporated to dryness, codistillated several times consecutively with pyridine, benzene and DMSO, and finally suspended in a known volume DMSO). The sealed mixture was shaken 24 hr at 37°.

After dilution with water (1:1), it was immediately applied to a 2×35 cm column of DEAE-Sephadex (formate). The elution was performed with a 6 l gradient of 0.1 M–1.0 M ammonium formate at pH 3.8. The ppGp! was eluted at 0.65 M, and pppGp! was eluted at 0.85 M.

The nucleoside phosphates of the three other bases came out 5%–10% earlier than the guanine series.

The pooled tetraphosphate fractions were lyophilized several times at 40° and 10^{-5} mm Hg. The residue was dissolved in 0.5 N KOH (25 ml) and incubated during three hr at 55°. The reaction mixture was slowly adjusted to pH 7.0 with 4 N $HClO_4$.

For analytical purposes, the nucleotide was precipitated as the barium salt. Chromatographic controls showed no breakdown during these manipulations.

The yield of guanosine tetraphosphate (pppGp)

was 75% and the ratio guanosine : phosphorus [14] was found to be 1 : 3.92 (calculated ratio, 1 : 4). The ultraviolet spectrum (pH 7.0) showed λ_{max} 252 mμ and λ_{min} 223 mμ O.D.$_{280}$/O.D.$_{260}$ = 0.60, O.D.$_{250}$/O.D.$_{260}$ = 1.10.

The nucleoside tetraphosphates of the three other bases give the expected analytical results and ultraviolet spectra.

Chromatographic data of the different phosphates are given in table 1.

For the enzymatic identifications the pppNp was incubated with bacterial alkaline phosphatase (4 μg per 10 O.D.λ_{max} units, 0.5 N tris buffer pH 8.5) and the products formed as a function of time were identified by paper chromatography.

ppNp was formed first, afterwards appeared rather simultaneously, ppN, pNp and pN. Prolonged incubation always resulted in complete breakdown to the corresponding nucleoside. In a parallel run, pppN was digested with this phosphatase. The time course for the appearance of ppN, pN and N was very similar to the breakdown of pppNp.

3. Discussion

By using standard techniques we were able to synthesize the four nucleoside 2′(3′)-phosphate 5′-triphosphates in 75% yield. These compounds, used as carriers, are of great help in the identification of the 5′ terminal of labelled natural RNA's [15] and in the determination of the number of 5′ terminals in the phage RNA replicative structures [16].

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