NSG-689

Condensation of Cytidylic Acid in the Presence of Polyphosphoric Acid

by

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In the press,

Biochimica et Biophysica Acta

SUMMARY

1. Cytidine-2'(3')-phosphate can be condensed in the presence of polyphosphoric acid.

2. The product was found to contain polyphosphate end groups which, however, could be removed by treatment with E. coli alkaline phosphatase (EC 3.1.3.1).

3. A single 2'(3')-P-terminal end group was found per chain.

4. With the exception of the free P-terminal end group, there was found to be one internally bound phosphate for every cytidine residue.

5. Although resistant linkages were detected, recovery of starting material from mild alkaline hydrolysis indicated that the linkages were predominantly 2'(3')-phosphodiester in nature.

6. The presence of 3'-phosphodiester linkages was demonstrated by hydrolysis with bovine pancreatic ribonuclease (EC 2.7.7.16).

7. The presence of a large proportion of 5'-phosphodiester linkages was demonstrated by hydrolysis with venom phosphodiesterase (EC 3.1.4.1).

INTRODUCTION

Preliminary publications have reported on the synthesis of phosphodiester linkages through the simple heating of cytidine-2'(3')-phosphate in the presence of polyphosphoric acid[1] and on the cocondensation of mixtures of cytidylic acid with other mononucleotides[2]. The present paper reports the partial structural characterization of the oligomericmixture obtained from cytidine-2'(3')-phosphate and polyphosphoric acid. The presence of a high proportion of phosphodiester linkages is demonstrated.

MATERIALS AND METHODS

Condensation of Cytidine-2'(3')-Phosphate

In order to obtain sufficient material for structural characterization, five condensations were carried out under identical conditions, and the purified products were pooled. In each case, 10 g of polyphosphoric acid*** and 5 g of cytidine-2'(3')-phosphate were mixed under nitrogen, and the mixture was heated at 65° for 1 h. After cooling, a magnetic stirring bar and a small quantity of water were added to the reaction flask, and the reaction mixture was dissolved by adding conc. NH_4OH. The flask was cooled during the addition by an ice-salt bath, and the pH was not permitted to rise above 7.0. The solution was transferred to a dialysis sac and was dialyzed against water in the cold for 3 days. The dialyzate was then absorbed on a large column or Dowex 1X8 chloride (2-3 lb) and, after washing with water, the product was eluted with 4 N formic acid in 0.5 N ammonium formate at 4°. The formate effluent was dialyzed against water for an additional 3 days in the cold and lyophilized to give the final product in 2.6% yield****.

Ultraviolet Spectra

Spectra were obtained in 0.01 N HCl and in 0.1 N NaOH on a Beckman DK2A ratio-recording spectrophotometer. Alkaline hyperchromicity was measured in 0.1 N NaOH after 48 h at 37°.

Elemental Analysis

Four samples of the product were dried over P_2O_5 *in vacuo* at room temperature for 24 h. The samples were analyzed for C, H, N and P, and an anhydrous formula weight was calculated for the average residue by setting the parts of carbon equal to 9.0.

Molecular Weight

Weight average molecular weight was determined from equilibrium sedimentation in a Spinco Model E analytical ultracentrifuge using Rayleigh interference optics[4].

Digestion with Alkaline Phosphatase and Ribonuclease

Incubation with *E. coli* alkaline phosphatase was employed in conjunction with an analysis for inorganic orthophosphate[5] to detect the presence of phosphomonoester end groups (or any

phosphate groups not involved in internucleotide linkage). The liberation of additional inorganic orthophosphate after addition of pancreatic ribonuclease to the incubation mixture was used as a measure of the proportion of 3'-phosphodiester linkages in the product.

In duplicate experiments, 5-6 mg of product was dissolved in 10-15 ml of 0.20 N sodium acetate (pH 5.3), and 0.5 mg of E. coli alkaline phosphatase was added. A second solution of identical concentration of product, but without phosphatase, served as a control for autohydrolysis. Enzyme blanks of 0.5 mg of alkaline phosphatase in 10 ml of buffer were also prepared, and the solutions were incubated at 37°. Aliquots were taken from all solutions periodically and were analyzed for inorganic phosphate. After maximal liberation of phosphate was observed, the experimental and enzyme blank solutions were divided into halves, 0.5 mg of pancreatic ribonuclease was added to one-half of each solution, and incubation was continued. Additional aliquots were taken from each pair of solutions and were analyzed for inorganic phosphate.

Hydrolysis in Alkali

Duplicate samples of the product were hydrolyzed in 0.10 N sodium hydroxide at 37° for 48 h. The hydrolyzates were adjusted to pH 8.0 with HCl and were fractionated on a 1 x 28 cm column of Dowex 1X4 formate. Cytidine-2'- and cytidine-3'-phosphates were obtained by elution with 0.2 N HCOOH. Hydrolysis after prior dephosphorylation with alkaline phosphatase was also studied.

End Group Analysis of a Dephosphorylated Preparation

In order to verify the presence of a single cytidine residue corresponding to the dephosphorylated P-terminal end group, duplicate samples of the oligomer were treated with E. coli alkaline phosphatase, and the dephosphorylated products were isolated on a 1 x 10 cm column of Dowex 1X4 chloride with 0.01 N HCl as the eluent. The dephosphorylated products were then hydrolyzed in 0.1 N NaOH at 37° for 48 h, and the hydrolyzates were fractionated on a 2 x 12 cm column of Dowex 50X4 (H^+). The column was eluted with water to remove anionic components, and then with 1 N HCl to remove cytidine.

Analysis for Labile Phosphate

In order to test for the presence of polyphosphate end groups, samples of product were heated at 80° in 1.0 N H_2SO_4, and aliquots of the solutions were periodically analyzed for inorganic phosphate.

Hydrolysis with Ribonuclease

Samples of the product (6-7 mg) were each dissolved in 1.0 ml of 0.20 N sodium acetate (pH 5.3), and 0.5 mg of pancreatic ribonuclease was added to each. The solutions were incubated at 37° for 4-12 h and were then transferred to a 1 x 28 cm column of Dowex 1X4 formate. After washing with water, the column was eluted with 0.02 N HCOOH.

Hydrolysis with Ribonuclease and Alkaline Phosphatase

Duplicate samples of the product (1.9-2.0 mg) were each dissolved in 0.5 ml of 0.20 N sodium acetate (pH 5.3) and 0.1 mg of E. coli alkaline phosphatase. Each solution was incubated at 37° for 12 h and then diluted to 2.0 ml and adjusted to 0.03 N in potassium chloride and 0.02 N in sodium borate. The solutions were then fractionated on a 1.2 x 8 cm column of Dowex 1X4 chloride, which had been prepared by washing with 0.03 N HCl in 0.02 N sodium borate. Cytidine was eluted from the column in the same solvent.

Hydrolysis with Venom Phosphodiesterase

Duplicate samples of dephosphorylated product (2 mg) were dissolved in 2.0 ml portions of 0.20 N sodium glycinate (pH 9.3) containing 0.025 N $MgCl_2$, and 0.1 mg of venom phosphodiesterase (Worthington) was added to each. The solutions were incubated at 37° for 3 and 4 h and were subsequently analyzed on a 1 x 27 cm column of Dowex 1X4 formate. After washing with water, cytidine-5'-phosphate was eluted in 0.02 N HCOOH.

RESULTS

The ultraviolet spectra were completely analogous to spectra obtained with previously reported preparations and indicated that phosphoramidate linkages did not participate in the internucleotide bonds[1,2].

Elemental analysis of the product revealed a ratio of 1.52 atoms of phosphorus for each nine-carbon cytidine residue (C, 26.2; H, 4.0; N, 10.6; P, 11.4). In other words, there is an "excess" of 0.52 phosphorus atom per cytidylic acid residue. On the basis of the elemental analysis, the anhydrous formula weight of the average cytidylic acid residue was taken at 350 (in agreement with the molar extinction coefficient). Since the molecular weight as determined by ultracentrifugation was 1960, the average chain length was 1960/350 = 5.6. An average chain of 5.6 residues will, therefore, have a total of 1.52 x 5.6 = 8.5 phosphorus atoms, of which 0.52 x 5.6 = 2.9 will be in excess of the single phosphorus per residue required for ideal phosphodiester linking. In addition, if we assume the presence of one phosphate end group per chain, we would expect to find 2.9 + 1.0 = 3.9 "external" phosphate groups (i.e., those not involved in the internucleotide linking). This would produce a phosphate end group analysis of 3.9/8.5 = 46% of the total phosphorus of the oligomer. This figure agrees with that determined by dephosphorylation of the oligomer with alkaline phosphatase (Table I). The conclusion drawn from this agreement is that the high proportion of "external" phosphate revealed by alkaline phosphatase treatment can be accounted for completely as the product of phosphorylation of the oligomer by the reagent polyphosphoric acid. Treatment of the oligomer with alkaline phosphatase thus removes only one end group per chain in addition to the "excess" phosphate. Therefore, the linkages of the oligomer must be entirely through phosphate groups, since other types of linkages (e.g., C-O-C chain branching) would produce more than one P-terminal end group per chain. Isolation of an alkaline phosphatase-treated fraction of the oligomer and subsequent alkaline hydrolysis produced one cytidine end group per 5.6 residues, in agreement with the conclusion of one P-terminal end group per chain (Table II).

The results of the hydrolysis of oligomer in dilute NaOH are tabulated in Table III. The yield of monomer increases from 58% to about 76% after dephosphorylation. This increase of 18% represents 0.18 x 5.6 = 1.0 residue. This observation and the fact that the "excess" phosphate was found to be entirely "external" led to the conclusion that the 2.9 "excess" phosphate groups per chain might be in the form of single polyphosphate end groups. This conclusion was verified by the analysis for labile phosphate (Table I). The 22.4% of the total phosphate which was liberated in 3 h corresponds to 1.9 phosphate groups per chain and is

precisely what would be expected from the hydrolysis of a statistical polyphosphate group of 2.9 units.

Figure 1 illustrates the effect of ribonuclease on the fractionation of the oligomeric mixture. The extent of hydrolysis to monomer units is tabulated in Table IV. The formation of cytidine-2'-phosphate indicates that the 2'(3')-terminal end group (P-terminal) of the oligomer is unsubstituted by "excess" phosphate. The 2'-isomer can only originate through the liberation of such end groups by hydrolysis of a penultimate 3'-phosphodiester linkage. The presence of cytidine-2'(3')-cyclic phosphate in the 4 h hydrolysis is not unexpected, since cytidine-2'-phosphate is known to inhibit the opening of the intermediate cyclic phosphate which is formed as the first step in the cleavage of phosphodiester bonds by ribonuclease[6]. Polyphosphates have also been shown to produce similar inhibition[7]. Apparently, extending the incubation to 12 h under these conditions results in the conversion of virtually all of the cyclic phosphate to cytidine-3'-phosphate, since the yield of the 3'-isomer plus cyclic phosphate remains constant. Dephosphorylation with alkaline phosphatase in conjunction to the ribonuclease digestion produced a 32% conversion to monomer. The simplification in structure which orcurs by removal of the polyphosphate end groups in this manner can be observed in Figure 2, which illustrates the fractionation of the dephosphorylated oligomeric mixture.

The dephosphorylated fraction which was hydrolyzed with venom phosphodiesterase amounted to 71% of the original sample. The yields of cytidine-5'-phosphate and cytidine from each of two hydrolyzates are tabulated in Table V. The presence of a small amount of cytidine is expected, since the enzyme preparation is known to contain a contaminating 5'-nucleotidase.

DISCUSSION

Preliminary studies on the products of the polyphosphoric acid-catalyzed polymerization of mononucleotides had suggested that these materials were extensively cross-linked because of the high proportion of apparent end groups (i.e., phosphate liberated by digestion with E. coli alkaline phosphatase)[1,2]. All of this "external" phosphate (in excess of the expected 2'(3') -end group) now is seen to reside in single polyphosphate termini which apparently are the legacy of the method of condensation. Since the 2'(3')-end of the chain was shown to carry the single, expected, phosphomonoester end group, these polyphosphate groups are probably located on 5'-hydroxyl ends. The presence of linkages other than through phosphate groups, therefore, is not consistent with the data. Resistance of some of the linkages to alkaline hydrolysis and to the action of venom phosphodiesterase, however, indicates that "non-natural" linkages or chain branches through 2'(3')-2'(3')-phosphodiester bonds are possible structural features of the product. However, 2'-5' and 3'-5' phosphodiester linkages must constitute the predominant mode of bonding. Although further work is necessary to clarify the total nature of the products obtained in this manner, it is clear that substantial quantities of phosphodiester bonds can be synthesized through the simple heating of cytidine-2'(3')-phosphate in polyphosphoric acid. The simplicity inherent in this method, the extensive phylogenetic evidence pointing to the role of polyphosphate in primitive organisms[8], and the interpretation of geochemical evidence[9-11] make polyphosphoric acid and its salts attractive materials[12,13] in the development of a theory of abiogenesis.

TABLE I

TYPES OF PHOSPHATE IN OLIGOCYTIDYLIC ACID

P_{total} (%)	P_1[a]	P_2[a]	P_3[a]	P_{labile} (%)
11.4	46-47	20-21	38-39	22.4

[a] P_1 = phosphate liberated by alkaline phosphatase, % of total

P_2 = phosphate liberated with RNAse, % of total

P_3 = % of phosphate linkages cleaved by RNAse $\left(\frac{P_2 \times 100}{100 - P_1}\right)$

TABLE II

END GROUP ANALYSIS OF DEPHOSPHORYLATED OLIGOCYTIDYLIC ACID

Sample	Cytidine Yield (%)	End Groups per 5.6 Residues
A	16.8	0.94
B	19.4	1.09

TABLE III

YIELD OF MONOMER AFTER ALKALINE HYDROLYSIS
OF OLIGOCYTIDYLIC ACID
BEFORE AND AFTER DEPHOSPHORYLATION

Sample	Before Dephosphorylation (% yield)	After Dephosphorylation (% yield)
A	57	--
B	59	--
C	--	79
D	--	73
E	--	76

TABLE IV

YIELDS OF CYTIDYLIC ACID FROM RIBONUCLEASE DIGESTION
OF OLIGOCYTIDYLIC ACID

Incubation (h)	Total Yield of Monomer (%)	Relative Yields of Isomers		
		3'-CMP (%)	2'-CMP (%)	2'(3')-cyclic CMP (%)
12	19	81	19	--
12	19	78	22	--
4	21	56	20	24

TABLE V

HYDROLYSIS OF DEPHOSPHORYLATED OLIGOCYTIDYLIC
ACID WITH VENOM PHOSPHODIESTERASE

Hydrolysis (h)	5'-CMP % Yield	Cytidine % Yield	Total % Yield
3	45	13	58
4	45	18	63

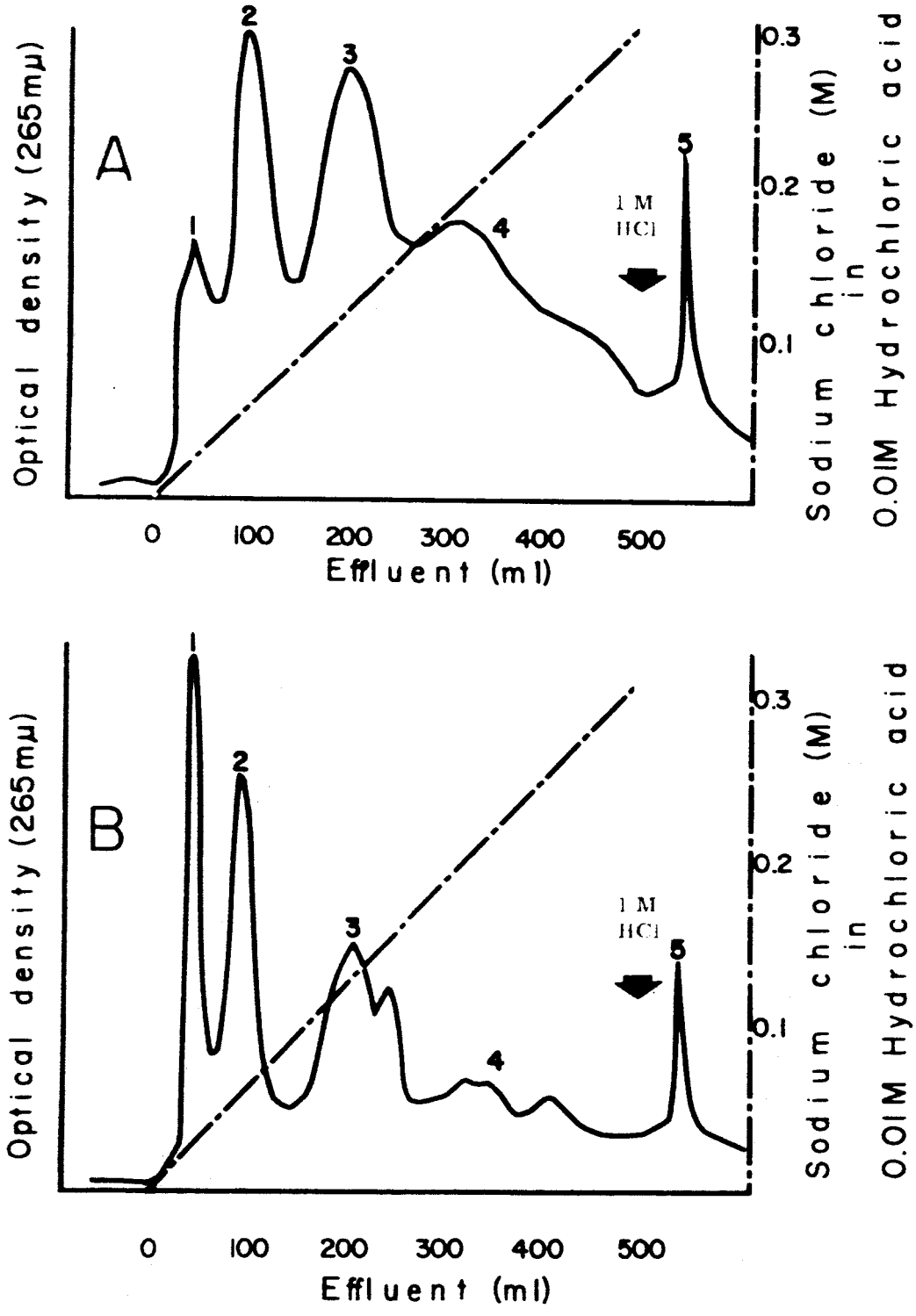

Fig. 1. Effect of ribonuclease on oligocytidylic acid (Dowex 1X4 chloride, 1.2 x 8 cm). A = before incubation, and B = after incubation with ribonuclease.

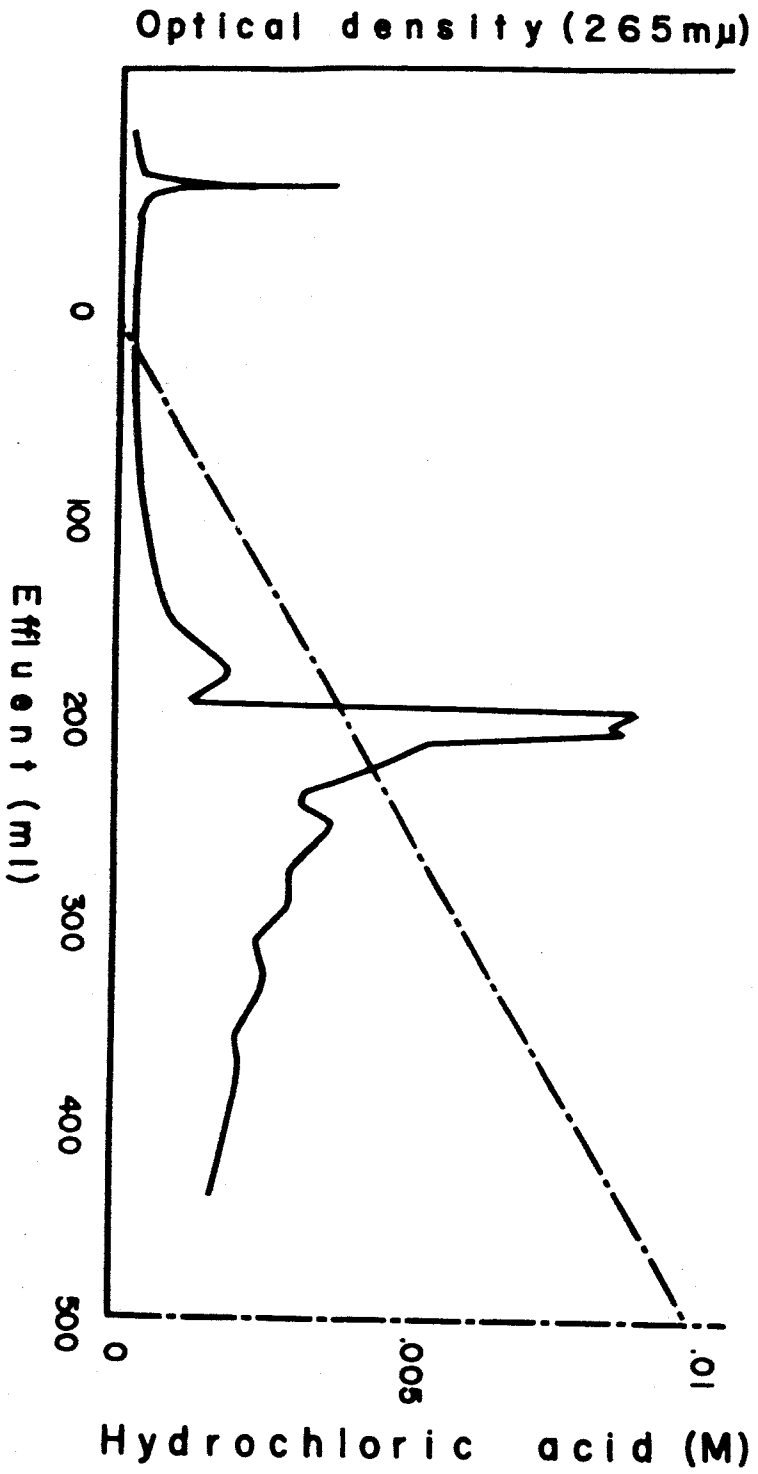

Fig. 2. Fractionation of alkaline phosphatase-treated oligocytidylic acid (Dowex 1X4 chloride, 1.2 x 8 cm).

FOOTNOTES

*Present address: Biomedical Research Group, Los Alamos Scientific Laboratory, University of California, Los Alamos, New Mexico. From the Ph.D. dissertation of Alan Schwartz, Florida State University, 1965.

**Contribution no. 047 of the Institute of Molecular Evolution. Research aided by Grant no. NsG-689 of the U.S. National Aeronautics and Space Administration.

***Polyphosphoric acid was a gift of the Victor Chemical Works and was found to correspond to 84-85% P_2O_5 on the basis of specific gravity and refractive index[3].

****Weight yields were based on the starting weight of nucleotide.

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