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Polymerization of the Cyclic Pyrophosphates of Nucleosides and Their Analogues

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Summary. When 2′-deoxythymidine-3′,5′-cyclic diphosphate, or the cyclic pyrophosphates of the acyclic nucleoside analogues II and IV are heated to 65–85°C in the presence of imidazole, oligomers with lengths up to 20–30 are formed in excellent yield. This reaction provides a useful source of oligomers for use as templates in aqueous condensation reactions. In the absence of evidence to the contrary, we assume that the oligomers are atactic. The potential significance of this reaction in prebiotic chemistry is discussed.

Key words: Nucleoside bis-monophosphates — Nucleoside analogues — Cyclic pyrophosphates — Ring-opening polymerization

Introduction

One of the main obstacles to the template-directed polymerization of many activated nucleoside-5′-phosphates and their analogues is the formation of the cyclic phosphates of the monomers (Hill et al. 1988). In general, the cyclic phosphates formed in these reactions, unlike the 2′,3′-cyclic phosphates of the nucleosides (Renz et al. 1971), are inert with respect to polymerization in solution, both in the presence and the absence of a template.

The activation of 3′,5′-bis-monophosphates and of their acyclic analogues, in a similar way, leads to the formation of cyclic pyrophosphates. In many cases the formation of the cyclic pyrophosphate is so efficient that polymerization is suppressed (Visscher and Schwartz 1988; Tohidi and Orgel 1989). As the bis-monophosphates of nucleoside analogues have been discussed as possible constituents of a genetic material more primitive than RNA (Joyce et al. 1987), it seemed interesting to determine whether or not cyclic pyrophosphates can be used as the substrates of polymerization under mild conditions. In this paper we report on efficient ring-opening polymerization of several cyclic pyrophosphates of nucleoside analogues in the presence of imidazole.

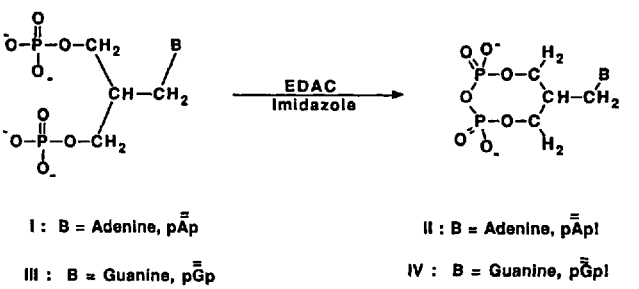
Materials and Methods

Materials. Reagent-grade chemicals were used without further purification. QAE-Sephadex Q-25-120 (anion exchanger), venom pyrophosphatase (type II), poly(U), poly(C), and Dowex-50W, 50X8-200 (hydrogen form) were purchased from Sigma Chemical Company, and alkaline phosphatase (calf intestine) from Boehringer Mannheim. 1,3-Propanediol diphosphate was a gift from Dr. A. Serianni, University of Notre Dame and Dr. R. Barker, Cornell University.

Ultraviolet (UV) spectra were recorded using a Beckman Model 35 spectrophotometer.

pĀp!. (II). A solution containing 0.01 M triethylammonium salt of 9-[3-hydroxy-2-(hydroxymethyl)prop-1-yl]-adenine diphosphate (pĀp) (I) (Tohidi and Orgel 1989), 0.1 M EDAC, and 0.1 M imidazole (pH 6.7) was stirred at room temperature.

Scheme I



Abbreviations: Im, imidazole; EDAC, 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide hydrochloride; EDTA, ethylenediaminetetraacetic acid; TEAB, triethylammonium bicarbonate; poly(C), polycytidylic acid; poly(U), polyuridylic acid; A, adenine; G, guanine; pĀp!, 9-[3-hydroxy-2-(hydroxymethyl)prop-1-yl]adenine cyclic diphosphate; pḠp!, 9-[3-hydroxy-2-(hydroxymethyl)prop-1-yl]guanine cyclic diphosphate; pTp!, 2′-deoxythymidine-3′,5′-cyclic diphosphate; pPrp!, 1,3-propanediol cyclic diphosphate

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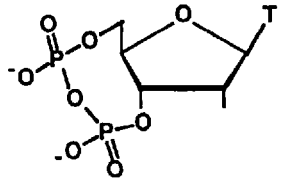
The course of the cyclization reaction was followed by TLC using *n*-propanol–ammonia–water (55:10:35) (system I) as eluent. After 2 days, the solvent was removed by evaporation and the residue dissolved in 0.05 M TEAB. The resulting solution was applied to a QAE-Sephadex anion exchange column (bead size 40–120), which was then washed with 0.05 M TEAB. The product was eluted using a gradient of 0.05–0.6 M TEAB. The fractions containing pĀp! were evaporated under reduced pressure. Coevaporation with toluene and then with methanol gave the triethylammonium salt of pĀp! as a white solid in 74% yield. The tetrasodium salt of pĀp! was prepared by passing 100 mg of its triethylammonium salt through a Dowex-50W, Na^+ form, column (mesh size 100–200) using water as eluent.

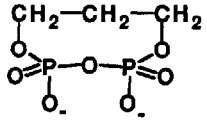
pḠp! (IV). pḠp! (IV) was prepared in 78% yield from pḠp (III) (Tohidi and Orgel 1989), following the procedures described above for the preparation of pĀp! (II).

pTp! (V). pTp! was prepared in 64% yield from 2'-deoxythymidine-3',5'-diphosphate, sodium salt, pTp, following the procedure described for preparation of pĀp!



V

pPrp! (VI). A solution containing 0.01 M of 1,3-propanediol diphosphate (cyclohexyl ammonium salt) (Hartman and Barker 1965), 0.1 M EDAC, and 0.1 M of imidazole (pH 6.7) was stirred at room temperature for 24 h. The course of the reaction was followed by TLC on the cellulose plates using MeOH : NH_4OH : H_2O (7:2:1) (system II) as solvent and the Hanes–Isherwood reagent as indicator (Hanes and Isherwood 1949). The solvent was then removed under reduced pressure and the resultant viscous oil was applied to a QAE-Sephadex column. The column was eluted using a gradient of 0.05–0.6 M TEAB buffer. Evaporation of the appropriate fractions, followed by coevaporation with toluene and MeOH, gave the triethylammonium salt of pPrp! as a white solid in 57% yield.



VI

Enzymatic Degradation of the Cyclic Diphosphates pĀp! (II), pḠp! (IV), and pPrp! (VI). Five ODs of pĀp!, pḠp!, or 200 μg pPrp! as the triethylammonium salt was added to 100 μl of Tris-HCl (0.2 M, pH 9.0) containing 0.04 M $MgCl_2$ and 0.2 units of venom pyrophosphatase (Type II). The solution was incubated at 37°C for 1 h. The course of degradation of pĀp! and pḠp! was followed by TLC using solvent system I. The degradation of pPrp! was followed in solvent system II. In attempted degradations using alkaline phosphatase, 5.0 ODs of pĀp! or pḠp! or 200 μg of pPrp! were added to 100 μl of Tris-HCl buffer (pH 8.0) containing 2 units of alkaline phosphatase. The solution was incubated at 37°C. The reactions were followed by TLC using the same solvent systems as mentioned above.

Reaction Mixtures and Chromatographic Analysis. Solutions were prepared that contained 0.01 M pĀp! (triethylammonium salt), and (1) 0.01 M HEPES buffer (pH 7.0), (2) 0.01 M imidazole (pH 6.7 with HCl), (3) 0.01 M $MgCl_2$, and (4) 0.01 M imidazole and 0.01 M $MgCl_2$. The mixtures were applied on microscope cover glasses in 60-μl aliquots. The samples were kept at different temperatures and humidities for 2–7 days. The relative air humidities of 100, 70, 37, 25, 19, 10, 5, and 0% at 85°C were maintained in desiccators containing aqueous sulfuric acid solution with densities of 1.0, 1.25, 1.4, 1.45, 1.50, 1.65, 1.68, and 1.84 (sulfuric acid concentrations of 0, 33.8, 50, 56, 60, 70, 86.5, and 100%). Reactions were also carried out in desiccators over P_2O_5 or solid NaOH. The samples were then taken up in dilute EDTA (20 μl, 0.1 M). Two μl of the resulting solution were used for HPLC analysis and the rest applied on paper and chromatographed in solvent system I for pĀp! and pḠp! or system II for pPrp!.

Reaction products from the UV-absorbing derivatives were analyzed by HPLC on an RPC-5 column with a linear gradient of $NaClO_4$ (0–0.04 M, in 60 min) in 0.02 M NaOH at a flow rate of 1.0 ml/min. The product distribution was determined by monitoring absorbance at 254 nm. The identity of the oligomeric products was established by showing that they cochromatographed with authentic samples (Tohidi and Orgel 1989). The efficiency of condensation to form dimers and higher oligomers was calculated from the appropriate integrated peak areas.

Paper chromatography was performed on Whatman 3 MM papers by the descending technique using (1) solvent system I (In order to obtain improved resolution of the oligophosphates we first applied a solution of 0.1 M EDTA K_4 at the origin, and then dried the paper. The reaction mixtures were then applied on the dried band.) or (2) solvent system II.

Oligomerization Reaction of pĀp! (Triethylammonium Salt) at Ambient Humidity. We prepared 60 μl of a solution containing 0.01 M of pĀp! and 0.01 M imidazole (pH 6.5, HCl), and heated it in a small petri dish on an oil bath at 85°C. After 2 days the dried spot was extracted as described above and analyzed by HPLC and paper chromatography.

Results

The bis-monophosphates pĀp (I), pḠp (II), pTp, and pPrp were cyclized efficiently in aqueous solution by a water-soluble carbodiimide to give cyclic pyrophosphates (Scheme I) in 74, 78, 64, and 57% yield, respectively. The identities of the cyclic pyrophosphates were established by showing that they were unaffected by alkaline phosphatase, but were hydrolyzed by venom phosphodiesterase to give material that cochromatographed with the starting nucleoside bis-monophosphates. The products formed by degradation with venom phosphodiesterase were completely dephosphorylated by alkaline phosphatase to give the corresponding bis-hydroxy compounds.

In preliminary heating experiments using pĀp! and pḠp!, analysis by paper chromatography established that no oligomers were obtained in the absence of imidazole and that, in the presence of imidazole, $MgCl_2$ prevented the formation of detectable amounts of oligomers longer than the dimer. Also the reaction with HEPES buffer or $MgCl_2$ gave no

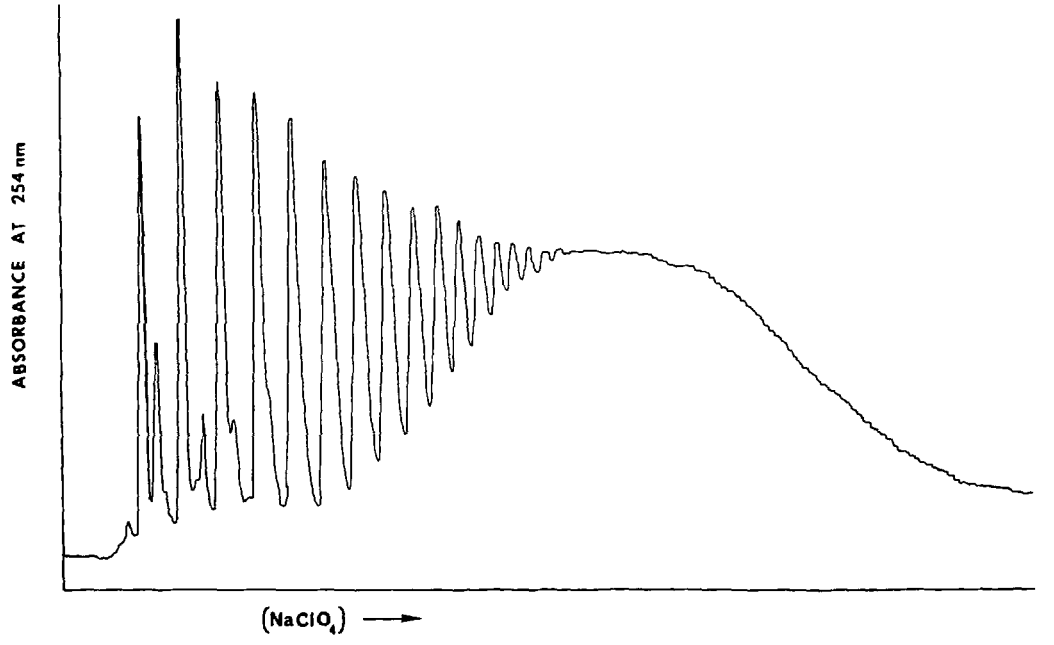


Fig. 1. Chromatographic analysis of products from a reaction mixture containing 0.01 M pÄp! (TEA salt) and 0.01 M imidazole after heating at 85°C over P_2O_5 for 2 days. Analysis of RPC-5 in 0.02 M NaOH with a linear gradient of $NaClO_4$ (0-0.04 M over 60 min) at a flow rate of 1.0 ml/min. Peak detection was by absorbance monitoring at 254 nm.

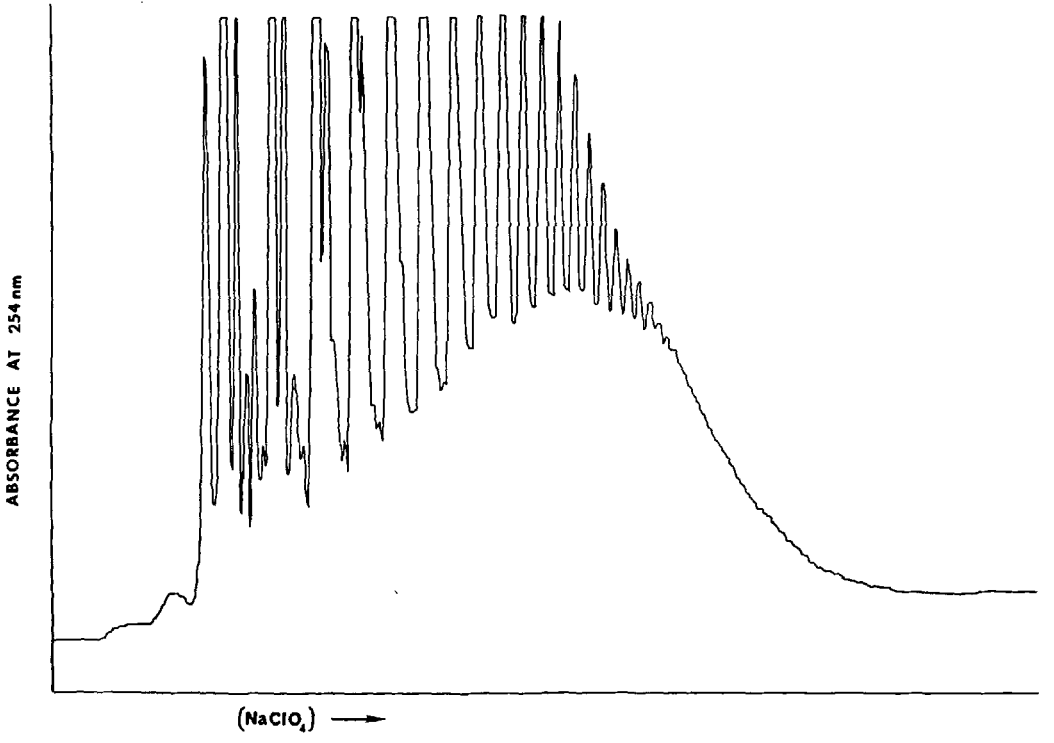


Fig. 2. Chromatographic analysis of products from a reaction mixture containing 0.01 M pḠp! and 0.01 M imidazole after heating at 85°C over P_2O_5 for 2 days. Conditions for chromatography as in Fig. 1.

detectable oligomers. Subsequent experiments used solutions containing 0.01 M substrate and 0.01 M imidazole (pH 6.7).

Both the yield and the rate of formation of products from pÄp! and pḠp! increased as the humidity decreased or the temperature increased. At humidities greater than 37% (50% H_2SO_4), after 2 days, no oligomers were observed at any temperature, but the starting materials were gradually converted to the open-chain bis-monophosphates. At 37% or 25%

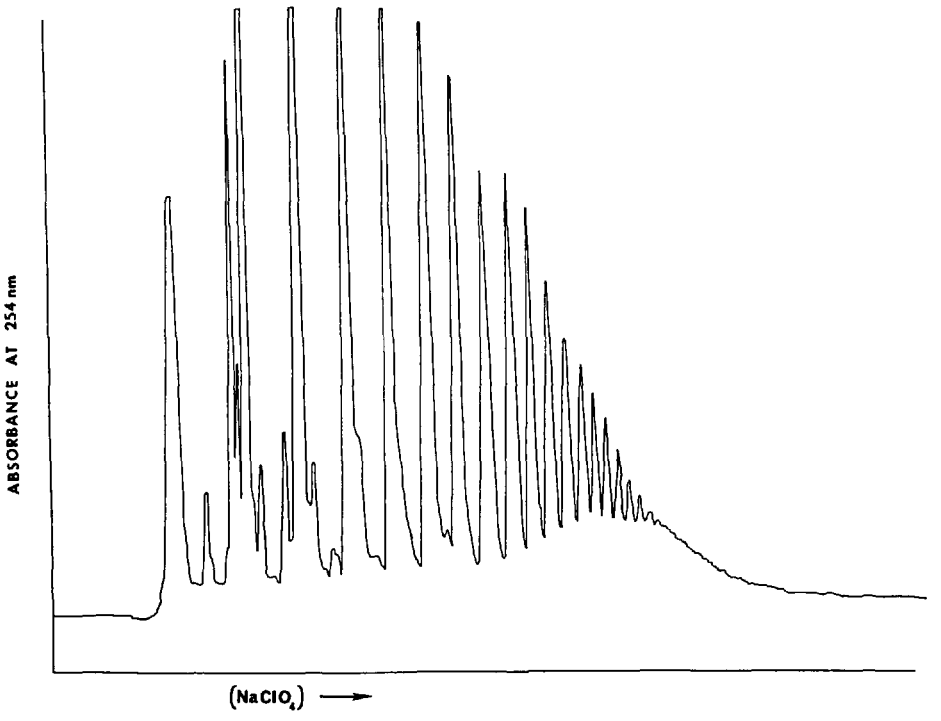


Fig. 3. Chromatographic analysis of products from a reaction mixture containing 0.01 M pTp! (TEA salt) and 0.01 M imidazole after heating at 85°C over P_2O_5 for 2 days. Conditions for chromatography as in Fig. 1.

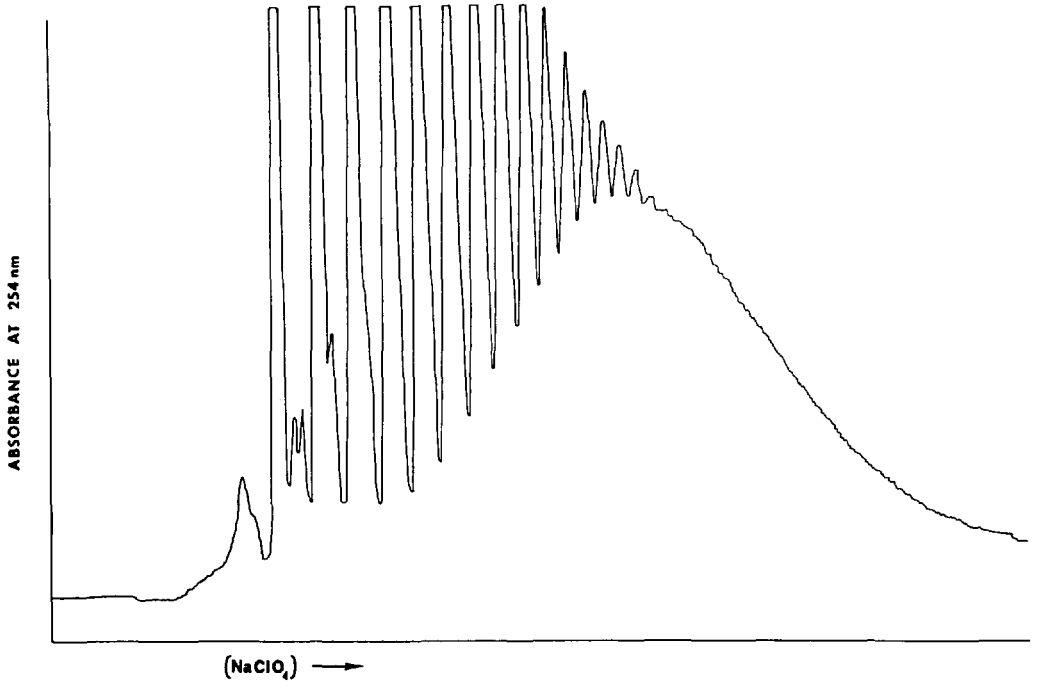


Fig. 4. Chromatographic analysis of products from a reaction mixture containing 0.01 M pĀp! (TEA salt) and 0.01 M imidazole after heating at 85°C over H_2SO_4 (concentrated) for 2 days. Conditions for chromatography as in Fig. 1.

humidity (50% or 56% H_2SO_4) in the temperature range 65–85°C small amounts of oligomers of pĀp! and pḠp!, up to the tetramer, were detected.

Paper chromatographic analysis showed that substantial yields of longer oligomers were obtained within 2 days at humidities in the range 0–19% (conc. 100–60% H_2SO_4) and temperatures from 65 to 85°C. Subsequently, at the higher humidities, oligomers tended to hydrolyze. After 7 days at a humidity of 10% (70% H_2SO_4), for example, the yield of longer oligomers had declined from 75% (2 days) to 59%.

HPLC analysis was used to study the detailed compositions of the product mixture under some of the more favorable conditions. The highest yields of longer oligomers were obtained at very low humidities over P_2O_5 at 85°C (Figs. 1–3). The longest

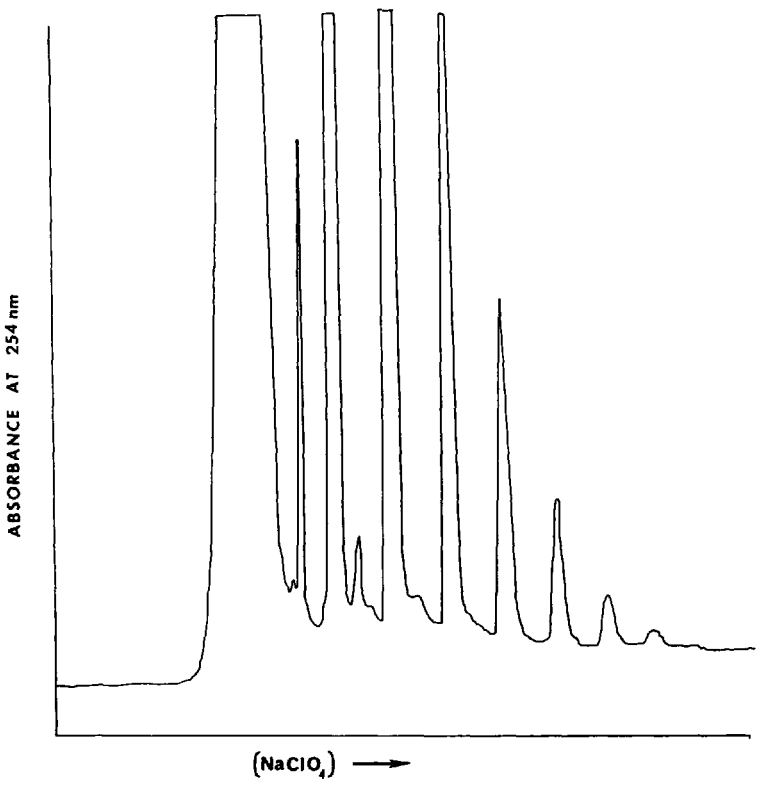


Fig. 5. Chromatographic analysis of products from a reaction mixture containing 0.01 M pĀp! (TEA salt) and 0.01 M imidazole after heating at 85°C over 70% H_2SO_4 for 2 days. Conditions for chromatography as in Fig. 1.

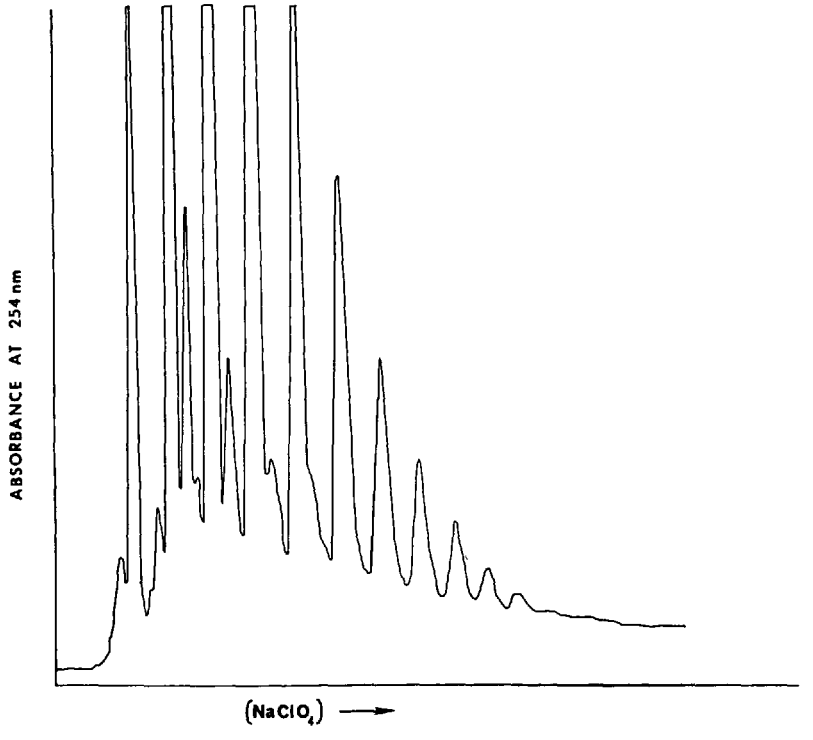


Fig. 6. Chromatographic analysis of products from a reaction mixture containing 0.01 M pĀp! (Na^+ salt) and 0.01 M imidazole after heating at 85°C over P_2O_5 for 2 days. Conditions for chromatography as in Fig. 1.

resolved oligomers correspond to 15–18-, 20–23-, and 18–21mers for pĀp!, pḠp!, and pTp!, respectively. However, it is clear that much longer oligomers are present in the unresolved tail in every case.

We also studied the oligomerization reactions of pĀp!, pḠp!, and pTp! over various concentrations of H_2SO_4 at temperatures in the range 65–85°C. The results obtained with concentrated sulfuric acid were very similar to those for the P_2O_5 reaction but with somewhat lower yield (Fig. 4). When we used less concentrated H_2SO_4 (humidities of 10% and 5%; densities of 1.65 and 1.68, respectively), much less efficient oligomerization was observed. For example

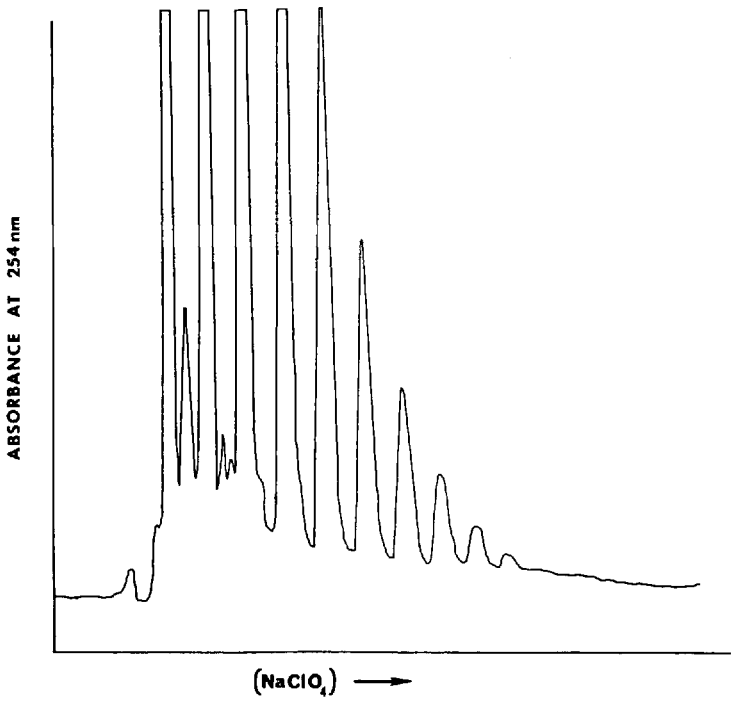


Fig. 7. Chromatographic analysis of products from a reaction mixture containing 0.01 M pĀp! (TEA salt) and 0.01 M imidazole, oil bath, after heating at 85°C for 2 days. Conditions for chromatography as in Fig. 1.

for pĀp! over H_2SO_4 (density = 1.65, 10% humidity) at 65°C, after 2 days oligomers up to 9mer were seen (Fig. 5).

In a few experiments we replaced the triethylammonium salt of pĀ p! by the sodium salt. The yield of longer oligomers was substantially reduced (Fig. 6) (P_2O_5, 85°C, 2 days). Polymerization of the triethylammonium salt of pĀp! at ambient humidity (oil bath, 85°C) also gave a reduced yield of longer oligomers (Fig. 7). The presence of a poly(U) template had no effect on the yield of oligo(Ā)s obtained from pĀp! (data not shown).

These condensations were, for the most part, very efficient (Table 1). For reactions carried out in a desiccator over P_2O_5 at 85°C, for 2 days, the efficiencies for pĀp!, pḠp!, and pTp! were 96, 93, and 95%, respectively. Over concentrated H_2SO_4 the corresponding efficiencies were: pĀp! 93%, pḠp! 85%, and pTp! 93%. At higher humidities (dilute H_2SO_4) the efficiency dropped significantly: pĀp! in 86% H_2SO_4 (5% relative humidity), for example, condensed with an efficiency of 68%. The efficiency of condensation over P_2O_5 of the sodium salt of pĀp! was 94.5%, and the efficiency of the reaction at ambient humidity was 72%.

The oligomers of pPrp cannot be detected by UV absorption. We did not study the oligomerization of pPrp! in detail, but we did confirm, by paper chromatography that substantial amounts of short oligomers were obtained at 85°C over P_2O_5. In other experiments, which we have not reported in detail, we attempted to polymerize the 3′,5′-cyclic monophosphate of Ā. No oligomers were found at temperatures up to 85°C.

Discussion

The formation of polymers from cyclic pyrophosphates is a typical example of a ring-opening polymerization (Ivin and Saegusa 1984). The need to include imidazole in the system can be understood in two ways. First, imidazole might function as an acid–base catalyst, facilitating the attack of the phosphate group of a ring-opened substrate or of an oligomer on a cyclic pyrophosphate molecule (Scheme II).

Table 1. Efficiency of selected oligomerization reactions at 85°C after 2 days

Composition of reaction mixture	% starting material	% hydrolyzed monomer	Efficiency (% oligomers)
pĀp!:Im, P_2O_5, 0% relative humidity	1.5	2.5	96
pĀp!:Im, H_2SO_4 (conc.), 0% relative humidity	0.5	6.5	93
pĀp!:Im, H_2SO_4 (86%), 5% relative humidity	3	31	66
pĀp!(Na^+):Im, P_2O_5, 0% relative humidity	0	5.5	94.5
pĀp!:Im, oil bath, ambient humidity	0	28	72
pḠp!:Im, P_2O_5, 0% relative humidity	1.7	5	93
pḠp!:Im, H_2SO_4 (conc.), 0% relative humidity	4	11	85
pTp!:Im, P_2O_5	0	5	95
pTp!:Im, H_2SO_4 (conc.)	0	7	93

Scheme II

$$p\bar{\bar{A}}p + p\bar{\bar{A}}p! \longrightarrow p\bar{\bar{A}}pp\bar{\bar{A}}p$$

$$p\bar{\bar{A}}p(p\bar{\bar{A}}p)_{n-2}p\bar{\bar{A}}p + p\bar{\bar{A}}p! \longrightarrow p\bar{\bar{A}}p(p\bar{\bar{A}}p)_{n-1}p\bar{\bar{A}}p$$

Second, the attack of imidazole on the cyclic pyrophosphate might generate a small equilibrium concentration of the monophosphorimidazolide, which might then undergo polymerization (Scheme III).

Scheme III

$$p\bar{\bar{A}}p! + Im \longleftrightarrow Imp\bar{\bar{A}}p$$

$$(n+1)Imp\bar{\bar{A}}p \longrightarrow \begin{matrix} Imp\bar{\bar{A}}p(p\bar{\bar{A}}p)_n + nIm \\ \text{or} \\ p\bar{\bar{A}}p(p\bar{\bar{A}}p)_n + (n+1)\,Im \end{matrix}$$

It is known that, under conditions similar to those used in the present experiments, amines including imidazole attack molecules containing a pyrophosphate bond, for example ATP and AppA, to generate phosphoramidates in substantial yield (Lohrmann 1977). We believe, therefore, that Scheme III provides the more plausible explanation of the efficient polymerization reactions that we have observed.

The detailed stereochemistry of the oligomeric products of these reactions cannot be deduced from our experimental results. In the absence of evidence to the contrary, we suppose that they are atactic, or at least far from stereoregular.

In the context of prebiotic chemistry, our procedure provides a useful method for generating nonstereoregular oligomers for experiments on template-directed condensation. We will, for example, attempt the template-directed oligomerization of various G derivatives, including $p\bar{G}p$, on templates derived by the ring-opening polymerization of $p\bar{C}p!$.

The relevance of this chemistry to events that may have occurred on the primitive earth is more problematical. It is unclear whether any prebiotic environment would have provided the high temperature, low humidity, and the abundance of imidazole that are needed to force the ring-opening oligomerizations to completion.

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