GENERATION OF LONG RNA CHAINS IN WATER

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The synthesis of RNA chains from 3’,5’-cyclic AMP and 3’,5’-cyclic GMP was observed. The RNA chains formed in water, at moderate temperatures (40 to 90°C), in the absence of enzymes or inorganic catalysts. As determined by RNases analyses, the bonds formed were canonical 3’,5’-phosphodiester bonds.

The polymerizations are based on two reactions not previously described: (1) oligomerization of 3’, 5’-cGMP to ~25 nucleotides-long RNA molecules, and of 3’,5’-cAMP to 4-to-8 nucleotides-long molecules. Oligo As further extended by reciprocal terminal ligation to yield RNA molecules up to more than 120 nucleotides-long. (2) Chain extension by terminal ligation of newly polymerized products of 3’,5’-cGMP on preformed oligonucleotides.

The enzyme- and template-independent synthesis of long oligomers in water from prebiotically affordable precursors approaches the concept of spontaneous generation of (pre)genetic information.

INTRODUCTION

The origin of informational polymers is not understood. The RNA polymerization process has been studied for five decades, the results showing that from preactivated precursors polymers of several tens can be obtained, as reviewed (1). These pioneering studies provide the proof-of-principle that RNA precursors can self-assemble yielding linear polymers. However, the prebiotic validity of a process based on complex preactivation procedures is limited (1, 2) and the problem of defining a prebiotically plausible chemical and thermodynamic scenario for the synthesis and accumulation of informational polymers remains open. The core of the problem is the standard state Gibbs free energy change (3,4) stating that condensation reactions are very inefficient in water. Given that extant polymerizations occur in water, this is a major difficulty, only partially solved by the fact that these processes at present occur inside the active site of enzymes where water activity may be drastically reduced. The other part of the extant solution, fruit of evolution, is the use of biologically highly preactivated triphosphate nucleotides (3). In primordia, RNA molecules had no enzymes to catalyze their chain-wise growth, and highly activated precursors can be considered as prebiotic only with difficulty.

We reasoned that for a preenzymatic polymerization to occur the solution must have relied on a simple and robust process. Ideally, such a process should have been based on compounds that were reactive yet relatively stable, chemically not too elaborate in order to allow their efficient production, and not too dissimilar from the products of their polymerization in order to minimize the chemical cost of the process.

It was observed that phosphorylation of nucleosides occurs in formamide simply in the presence of a source of organic or inorganic phosphate at temperatures at which both the reactants and the products are stable (5). Phosphorylation occurs in every possible position.
of the nucleoside sugar moiety resulting, both for purine and pyrimidine nucleosides, in the production of 2’,3’,5’,2’,3’-cyclic and 3’,5’-cyclic XMPs (5). The phosphorylation reaction is faster for the open than for the cyclic forms, while higher stability of the cyclic forms at higher temperature favours their accumulation.

Coupled with the facile synthesis of all the nucleic bases from formamide (6) and with the formation of acyclonucleosides by TiO₂-catalyzed formamide photochemistry (7), the nonenzymatic phosphorylation of nucleosides (5) shows that the formation of cyclic monophosphate nucleosides is chemically simple and prebiotically plausible. The formation of both 2’,3’ and 3’,5’-cyclic XMPs in water starting from nucleosides and an inorganic source was also observed (8).

The unsophisticated chemistry required for the formation of both open and cyclic nucleotides prompted us to investigate the possibility of their spontaneous polymerization. If so, nonenzymatic (pre)genetic polymerization could have taken place in warm little pond conditions, close to those imagined by Darwin (9).

EXPERIMENTAL PROCEDURES

Materials

Adenine, adenosine, adenosine 2’-monophosphate (2’AMP), adenosine 3’-monophosphate (3’AMP), adenosine 5’-monophosphate (5’AMP), adenosine 2’,3’-cyclic monophosphate (2’,3’-cAMP), adenosine 3’,5’-cyclic monophosphate (3’,5’-cAMP), adenosine 5’-diphosphate (ADP), adenosine 5’-triphosphate (ATP), guanosine 3’,5’-cyclic monophosphate (3’,5’-cGMP), cytosine 3’,5’-cyclic monophosphate (3’,5’-cCMP), uridine 3’,5’-cyclic monophosphate (3’,5’-cUMP) were from Sigma Aldrich, analytical grade.

Oligonucleotides. The oligonucleotides 5’A₂₄₃’, 5’C₂₄₃’, 5’A₁₂C₁₂₃’, 5’A₁₂U₁₂₃’, 5’U₂₄₃’, 5’G₂₄₃’ were purchased from Dharmacon and were provided unphosphorylated, at both the 5’ and 3’ extremities.

Methods

Polymerization protocols and analysis — Concentrated solutions of the appropriate nucleotide (2’AMP, 3’AMP, 5’AMP, 2’,3’-cAMP, 3’,5’-cAMP, 3’,5’-cGMP, 3’,5’-cUMP, 3’,5’-cCMP) were diluted in water to the wanted final concentration. Concentrations between 1 μM and 0.1 M were analyzed. Temperatures between 25 and 90°C and pH values 3.2, 3.7, 5.0, 5.4, 6.1, 8.0, 8.2 and 8.4, obtained by TrisHCl-buffering of distilled deionized MilliQ water, were tested. Other variables are discussed where appropriate.

Acrylamide gel electrophoresis — Standard methodologies were used, with the following specifications: (1) 12 % polyacrylamide was used in analyses encompassing the whole product of the polymerization reaction, from the ³²P-labelled monomer to the highest molecular weight fragments (> 100 units). (2) Longer runs on 16% polyacrylamide gels were used for the analysis of low molecular weight polymers.

With sequences allowing good resolution, the average chain length (Navg) of the oligomers was determined by the equation

\[
N_{\text{avg}} = \frac{\sum n_i N_i}{\sum n_i}
\]

where \(n_i\) is the number of chain (in %) and \(N_i\) is the length of RNA chains in nucleotides.

Reference ladders — The nucleotide ladders used as standard in the gel electrophoretic analyses of the polymerization products consisted of partially hydrolyzed 24mer PolyG or PolyA (Dharmacon), as appropriate. Products of combinatorial ligation of preformed oligonucleotides were also used as markers, obtained as detailed in (10). In practice, labelled 5’A₂₄₃’ was mixed with unlabelled 5’A₁₂₃’ yielding 39, 48, 69, 78 and 96 nucleotides-long fragments.

For details, handling and analysis of the RNA hydrolytic products see (11). In brief, terminally labelled RNA oligonucleotides were hydrolyzed in water at 90°C for different time periods (between 0 and 24 hours) and pre-analyzed on polyacrylamide gel.

Terminal labelling of the material polymerized from unlabelled cyclic nucleotides — The products of the polymerization reactions from cyclic nucleotides were ethanol-precipitated and dissolved in 44 μl of water. For de-
phosphorylation, 1 μl of shrimp alkaline phosphatase (CIAP, 1 U/μl, MBI Fermentas) was added along with 5 μl of 10× CIAP buffer, and the reaction was incubated at 37°C for 30 min, followed by phenol extraction and ethanol precipitation. Glycogen (1 μl of stock 20 mg/ml) was added to facilitate precipitation. RNA was pelleted by centrifugation, then dissolved in 16 μl of water and labeled at the 5′-termini with 32P. 

Phosphorylation was carried out by adding 1 μl of T4 polynucleotide kinase (T4 PNK, 10 U/μl, New England Biolabs), 2 μl of 10× PNK buffer and 0.5 μl [γ-32P]ATP, followed by incubation at 37°C for 30 min. For gel electrophoresis, 10 μl aliquots of the RNA samples were resuspended in 100% formamide and separated by electrophoresis on 12 or 16% polyacrylamide gels containing 7 M urea, along with the indicated markers.

**RESULTS**

The oligomerization capacity of the cyclic forms (2′-3′ or 3′-5′) of the four monophosphate nucleosides guanosine, adenosine, cytidine and uridine was tested. The open nucleotides 5′AMP, 3′AMP, 2′AMP were also tested in water at temperatures between 40 and 90°C. A number of additional variables were analyzed: concentration, time, addition of formamide (from 0 to 100%), presence of several minerals known to catalyze phosphorylation (5) or to increase the half-life of nucleic polymers (11, 13-15), addition of Na4P2O7 or Na5P3O10, combinations thereof. Of all the conditions tested, the simplest proved to be the best: water between 40 and 90°C. Several pH values (3.2, 3.7, 5.0, 5.4, 6.1, 8.0, 8.2, 8.4) were tested. The results observed were marginally different. The afforded polymers were 5′ terminally labelled with [γ-32P] ATP by T4 polynucleotide kinase and the products were characterized by gel electrophoresis, allowing detailed evaluation of the lower-sized oligomers.

**Syntheses from open nucleotides.**

No product of polymerization was observed upon incubation of 2′AMP or 3′AMP in water (nor in any of the reaction variants listed above) at temperatures encompassed between 40 and 90°C for periods up to 400 hours. Only degradation of the input nucleotides was observed (data not shown). 5′AMP afforded only traces of oligomerized compounds whose total did not exceed 0.5% of the input (data not shown). The short half-life of 5′AMP at 90°C (35 hrs) (11) is not compatible with the possibility of accumulating oligomers.

**Syntheses from cyclic nucleotides.**

3′,5′-cGMP.

Fig. 1 shows the products of polymerization obtained by treating 3′,5′-cGMP in water. The formation of oligomers is evident. 3′,5′-cGMP polymerized into RNA chains that reached a size of at least 25 nucleotides, the predominant oligomer being the 8mer. Panel A reports the synthesis obtained at 85°C as a function of the 3′,5′-cGMP concentration, showing that above the optimal concentration of 1mM, chain elongation is impaired and the preferentially formed 8mer accumulates. Panels B and C show the syntheses obtained at the optimal 1 mM and at the highest possible (before aggregation) 100 mM concentration as a function of the temperature. In
both cases the highest temperature tested was the most favourable for chain extension. Below 60°C the reaction rate dropped rapidly (data not shown).

The oligomers shown are the products of synthetic reactions lasting 1 hour. In kinetic analyses it was observed that at the optimal concentration (1 mM) synthesis was fast, a Navg of 11.8 being reached during handling time (< 1 min), followed by slow stepwise further growth. The kinetic constant of this further growth was determined by measuring the Navg of the Oligo G chains formed as a function of time at 85°C with 1 mM 3',5'-cGMP and was 0.4 x h⁻¹.

3',5'-cAMP.

Under the same conditions of the 3',5'-cGMP polymerization, 3',5'-cAMP polymerized by a two-steps mechanism. Fig. 2 shows the two steps observed in a 3',5'-cAMP-fed growth experiment. First, a family of short oligomers was synthesized rapidly. The steady-state Navg of 5.32 (Fig. 2, lane 1) was reached by 60 min (50% of molecules formed in 20 min). The kinetic constant of the reaction leading to the formation of the short Oligo A molecules (Navg 5.32) was determined at 85°C and was 2 x h⁻¹. The short oligomers did not continue growing by slow ladder-wise addition, as for 3',5'-cGMP, but extended their size forming a heterogeneous population (Fig. 2, lane 3) in which a rapidly formed 16mer was prominent. Sequence extension lasted 200 hours, forming molecules >100 nucleotides long (Fig. 2, lane 4). The distribution of the products of oligomerization beyond 28 nucleotides in length was size-discontinuous (see the numbering at the side of lane 4), comprising a complex series of fragments. Such heterogeneous numerical distribution is best interpreted as the result of ligation of shorter pieces. A model study (10) showed that mixing a limited number of different RNA oligomers in water yields a complex population of differently sized RNA fragments by nonenzymatic ligation. This second reaction, presumably based on ligation of the components of a heterogeneous population, is too complex to allow calculation of kinetic constants. By contrast, 2',3'-cAMP yielded only short oligomers, up to tetramers (data not shown).

Polymerization of 3',5'-cUMP and 3',5'-cCMP yielded only short fragments (Navg 5.49 and 5.45 respectively) at 85°C, which did not grow further.

The bonds formed, as determined by RNase analyses.

The type of phosphate bond formed in the polymers derived from 3',5'-cGMP and 3',5'-cAMP was analyzed by enzymatic digestion with Snake Venom PhosphoDiesterase I (EC 3.1.4.1, a 5'-exonuclease cleaving 3'-5' and 2'-5' phosphodiester bonds from the 3' extremity in a nonprocessive manner) and with P1 endonuclease (EC 3.1.30.1, a 3'-5'-specific ribonuclease). Treatment of the products of polymerization with 1 mU of SVPD I or of P1 for 20 min at 37°C completely converted the oligos into monomers, showing that the bonds formed are canonical 3'-5' phosphodiester bonds (data not shown). For details of these RNase assays, see (10). The type of phosphate bond formed in the Oligo G was further analyzed, as described below, confirming the formation of 3'-5' bonds.

On the mechanism of polymerization.

Although detailed mechanistic aspects of the observed polymerization of cyclic nucleotides are beyond the aim of the present communication, the following facts elucidate the basics of the reaction:

i) the RNase digestion assays mentioned above show that the bonds formed by polymerization of 3',5'-cyclic nucleosides are standard 3'-5' phosphodiester bonds. Given that the starting monomers are 3',5'-cyclic phosphates, this is not unexpected. The combined SVPD I and P1 RNase analyses rule out the formation of 2'-5' bonds, of pyrophosphate bonds, or more complex alternatives.

ii) 3',5'-cyclic nucleoside monophosphates hydrolyze in water yielding (in the temperature and pH conditions in which polymerization occurs) a mixture of 5' and 3' monophosphates, as verified by HPLC (data not shown), and as originally reported (16).

Thus, the polymerization could occur according to two different alternative models:

model A: the reactive species is a 5’XMP afforded by the opening of the 3’ phosphoester bond of the cyclic nucleotide. In this case, polymerization would occur via the 5’ phosphate reacting with the 3’ OH of another 5’XMP, as indicated by the spark symbol in Fig. 3. The reactive species is a 3’ XMP and the polymerization occurs via the 3’ phosphate reacting with the unphosphorylated 5’ extremity of another 3’XMP molecule. Model A would...
lead to the phosphate group being on the top sugar molecule (as shown in Fig. 3), rather than on the lower sugar molecule (model B, not shown).

The bias would be solved in favour of model A if neo-formed Oligo G, obtained as described in Fig. 1, would ligate to the 3’ non-phosphorylated extremity of an acceptor oligo through 3’-5’ phosphodiester bonds (as schematically described in Fig. 5). The experiments reported below (Figs 4-6) show that this is the case: the neo-formed Oligo G ligated with 3’-5’ bonds to the 3’ OH extremity of a 5’C243’ and of a 5’A12C123’ oligomer. Thus, model A applies, as shown in Fig. 3.

In summary, in the presence of the thermodynamic driving force provided by stacking interaction, an isoenergetic phosphodiester exchange reaction is favoured, affording the observed products. The possibility that the reaction occurs by general acid-base catalysis is disfavoured by the observation that neither the 3’,5’-cAMP nor the 3’,5’-cGMP polymerizations are pH dependent (between pH 3.2 and 8.4, data not shown).

The fact that the order of the stacking potentials of the bases correlates with the corresponding polymerization rates (see below) establishes the relevance of stacking interactions in this reaction.

RNA chain extension.

Nonenzymatic ligation of nonenzymatically polymerized Oligo G to the 3’ extremity of preformed Oligo Cs.

Do cyclic nucleotides polymerize in the presence of preformed oligonucleotides? If so, is this condition interactive? The answer is positive, as described below. The following oligonucleotides were tested: 5’A243’, 5’C243’, 5’A12C123’, 5’A12U123’, 5’U243’, 5’G243’. Each one of these oligos was reacted with 3’,5’-cAMP, 3’,5’-cGMP, 3’,5’-cCMP, and 3’,5’-cUMP.

Fig. 4 Panel A shows the results of the reaction of 5’-labelled 5’C243’ with different concentrations of unlabeled 3’,5’-cAMP, 3’,5’-cGMP, 3’,5’-cCMP, and 3’,5’-cUMP, as indicated.

The key observation is that 3’,5’-cGMP actively reacted with the preformed oligo, affording longer fragments.

In particular: a group of molecules with a number average (Navg) of 42 formed in the presence of 3’-5’-cGMP (lanes 6-8), that grew up to an observed length of >50 nt in the presence of the higher concentration of cyclic nucleotide (as counted in the right corner inset, showing a lower exposure of the relevant gel position). A slower migration band is also observed in the upper part of the lanes 6-8 (asterisk), probably representing a dimeric form of the extended sequence.

The Navg was calculated from graphical extrapolation of gel positions in the appropriate autoradiographic exposures. The band-compression effect characteristic of the C residues prevents a better resolution of high molecular weight oligomers and a more precise evaluation of fragment lengths. The system was explored with higher precision in 5’A12C123’ polymers (see below).

All the 5’C243’ fragments covalently reacted with Oligo G oligos (lanes 7 and 8) and formed a new population reaching an average length of 42. This entails that in the solution in which the reaction takes place Oligo Cs and Oligo Gs interact, presumably by base-pairing, to form a double-strand. Double-strands withstand hydrolysis more than single-strands. If this occurs also in our conditions and sequence setup, a circa 18 bases-long footprint should be produced, which is actually observed (Fig. 4A, dots in lane 7; scheme on the right side). The open dots at the bottom of the lane indicate where the footprint, is not observed, showing that the chain extension does not occur from the 5’ extremity.

The following is also noted: (1) the C stretch is highly sensitive to hydrolytic degradation [as already reported (17)]. (2) 3’,5’-cAMP does not support polymerization growing on the 3’ extremity nor supports multimerization by ligation [as observed for 5’A243’oligos (10)]. Starting at 10 mM concentration, 3’,5’-cAMP enhances the hydrolytic degradation of the 5’C243’ oligonucleotide (lane 5). The same behaviour was observed on PolyA23U, PolyA24 and on PolyG24 (data not shown). (3) 3’,5’-cCMP and 3’,5’-cUMP are inert. Thus, only the reaction of Oligo C with 3’,5’-cGMP was explored further.

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Fig 4 panel B shows the RNA chain extension of 5’C243’ by 3’,5’-cGMP as a function of cyclic nucleotide concentration. Panel C shows
selected examples of the same reaction on 5′A12C123′.

Consistent with the calculated \( N_{av} \) of the Oligo G polymerized from 3′,5′-cGMP reported in Fig. 1 (in synthesis reactions in which the 8mer was prevailing), the family of Oligo Gs that polymerized from 3′,5′-cGMP in the presence of the 5′A12C123′ 24mers and that ligated to its 3′ C-extremity had a \( N_{av} \) of 8.75 (Fig. 4 Panel C). This \( N_{av} \) value was determined from the \( N_{av} \) calculated from the fragment sizes observed in the gel migration ladder (\( N_{av} = 32.75 \)) subtracting 24 (that is, the size of the acceptor 24mer oligo).

The following is also noted: the footprint on the C12 moiety is shorter relative to the one on the C24 oligo, as shorter are the chains produced (\( N_{av} = 32.75 \), corresponding to an extension of 8.75 on the 24mer and to a footprint \( \geq 8 \) residues, as indicated by dots) and as predicted in a model based on the PolyC - PolyG base pairing in water.

3′,5′-cAMP, 3′,5′-cCMP, and 3′,5′-cGMP did not support chain extension on the 5′A12C123′ (nor on the 5′C243′ - data not shown).

The pre-synthesized Oligo G did not bind to (nor 3′,5′-cGMP-fed polymerization occurred on) pre-synthesized PolyA oligos (data not shown), thus excluding that the 5′A extremity of the 5′A12C123′ molecule supported RNA chain extension on the polyC oligonucleotides.

The fact that a footprint is observed starting from the position in which sequence extension begins (i.e., the 3′ extremity) and is oriented in the specular direction, provides an assay for the presence of newly formed complementary sequences. No footprint is observed on the 5′ extremity, indicating that sequence extension only occurs on the 3′-OH extremity based on the 5′ P-group from the incoming molecule, and not vice versa.

A quantitative evaluation of the RNA chain extension occurring on 5′C243′ and on 5′A12C123′ as a function of the cyclic nucleotide concentration is reported in Fig. 4 Panel D. The plot shows that the growth of short segments occurring on 5′A12C123′ (\( N_{av} = 8.75 \)) levels off at lower concentration of 3′,5′-cGMP, relative to the growth on 5′C243′ (\( N_{av} = 18 \)).

The kinetic constant of the reactions leading to the formation of the extended monomers could not be determined because the reaction was too fast even at the lowest concentration tested (200 nM 5′C243′ and 1 \( \mu \)M 3′,5′-cGMP), at 40°C. Reaction Rates are given in Table 1.

In conclusion, 3′,5′-cGMP efficiently polymerizes in the presence of PolyC and is covalently bound to its 3′ extremity. Given that the 3′ extremity of the 5′A12C123′ oligo bears no phosphate but ends by OH in 3′, and given that the ligation occurred via 3′-5′ phosphodiester bonds (see below, section on RNases), the observed chain extension necessarily occurred by ligation through the 5′ phosphate group carried by the neo-polymerized Oligo G, as shown in Fig. 5.

**The rate limiting step.**

The rate limiting step of the polymerization reaction is the dinucleotide formation or the extension reaction? In the 3′,5′-cGMP system, in order to answer this question we tried to measure the kinetic constant of the dinucleotide formation by lowering the concentration of 3′,5′-cGMP down to the detection limit of the assay. The results, reported in Fig. 6, show that the shortest observed measurable chain is the G8 oligomer (\( N_{av} = 8.75 \)) and that its formation is immediate. The experiment also shows that the amount of elongated polymer formed depends on the concentration of 3′,5′-cGMP, not on a kinetically limiting step. Given that the kinetic constant of the elongation reaction, as determined in the same optimal conditions (85°C, 1mM 3′,5′-cGMP), is relatively low (0.4 x h\(^{-1}\)) the limiting step is chain elongation.

As for the 3′,5′-cAMP system, the kinetic constant for chain elongation is 2 x 10\(^{-1}\)h. The kinetics of formation of the dimer was followed by HPLC analysis of the polymerized products. At no time point the dimer was observed to accumulate relative to the trimer, tetramer, etc. showing that in this system the limiting step is the dimer formation.

**Characterization of the bond formed upon ligation of the 5′A12C123′ oligo with the neo-synthesized Oligo G.**

The 5′A12C123′ oligo was reacted with 3′,5′-cGMP (60°C, 6 hrs, 400 \( \mu \)M 3′,5′-cGMP), then treated with T1 or SVPD I ribonucleases. Fig. 7 shows that the 5′A12C123′G8,753′ is sensitive to the two nuclease, thus confirming the 3′-5′ nature of the phosphodiester bonds formed, both
in the Oligo G and between the Oligo G and the 5′A12C12 3′ oligomer.

A vast literature has accumulated on the preferential formation of the 3′-5′ over the 2′-5′ phosphodiester linkages or (more often) its contrary, in oligomerizations entailing nucleoside-5′-phosphorimidazolides and related phosphoramidates, or in carbodiimide-mediated ligations [reviewed in (1)]. The syntheses may preferentially form one type of linkage (i.e., the 2′-3′ linkage) (18) or the other (19), or both (20). For an in-depth review of this topic see (20, 21). The summary of our RNases analyses is that: in the oligomers formed from 3′,5′-cGMP and 3′,5′-cAMP the linkage is 3′-5′. The discrepancy with the fact that the 2′,3′ is the most commonly observed linkage in abiotic polymerizations from preactivated compounds may be explained simply by the fact that none of the previously reported syntheses was performed with 3′,5′cyclic nucleotides in water, as in our case.

**Increased stability of RNA oligonucleotides in water is caused by the presence of cyclic monophosphate nucleosides.**

The half-life of RNA oligonucleotides in water has been matter of detailed analyses (11, 17). As expected, and based on a large body of previous studies, the observed half-life of RNA molecules depends on sequence composition, temperature, pH and concentration. In the present analysis, the products of polymerization from 3′,5′-cGMP and 3′,5′-cAMP showed unexpectedly high t½ values. It was found that the increased life span of the oligos in water is induced by the presence of the free cyclic nucleotide, presumably due to interference with the hydrolytic degradation process by stacking interaction (Fig. 8).

**DISCUSSION**

**How RNA polymerization was started?**

A key step missing in the reconstruction of the origin of living systems is an abiotically plausible synthesis of RNA. In order to fill this gap, the robust synthesis and the simultaneous presence of all the necessary nucleic acid precursors [which is possible in principle (22)], an abiotic procedure for their activation, and a thermodynamically sound polymerization mechanism are needed.

In this logic we have analyzed nucleotide oligomerization in the conceivably simplest solvent and environment: water at temperatures between 40 and 90°C. In spite of the limits set in principle by the standard-state Gibbs free energy change problem (3,4), we observe that the process does actually take place in water and report the nonenzymatic formation of RNA chains in water from 3′,5′-cyclic nucleotides.

We describe three mechanisms for nonenzymatic RNA generation: RNA polymerization from monomers, RNA ligation, RNA extension by polymerization on pre-existing oligomers and ligation. RNA ligation was recently reported in a model study performed on PolyA oligomers (10).

We observe that 3′,5′-cGMP polymerized into RNA chains at least 25 nucleotides long (Fig. 1), the predominant oligomer being the 8mer. At the optimal 1 mM concentration, synthesis was fast, a N_avg of 11.8 being reached within 1 min, followed by slow stepwise further growth. Canonical 3′,5′-phosphodiester bonds were formed, as determined by RNase sensitivity. 3′,5′-cAMP polymerized more slowly to oligomers that reached a N_avg of 5.32 within 1 h. These oligomers expanded their size by inter-fragments ligation for a period of at least 200 hrs, yielding molecules > 100 nucleotides long.

**The plausibility of 3′,5′ cyclic nucleotides as precursors in nonenzymatic polymerizations**

Nonenzymatic polymerizations require pre-activated monomers (3,4). The results obtained with the phosphoramidated nucleotides commonly used (23, 24-30) show that the accumulation of polymerized forms is possible once suitable activated monomers are available. Although these studies provide useful data on the formation and properties of RNA oligomers formed by chemical synthesis, their prebiotic relevance was questioned (1, 2). The action of several organic agents (31-33) and of inorganic polyphosphates (34) on polymerization in aqueous solution was reported.

An innovative nonenzymatic polymerization system was recently reported describing the lipid-assisted synthesis of RNA-like polymers from mononucleotides (35). Chemical activation of the mononucleotides was not required. Instead, synthesis of phosphodiester bonds was driven by the chemical potential of fluctuating anhydrous and hydrated conditions, with heat providing the activation energy. Chemical complexity prevented the full analysis of
the RNA-like products of this otherwise promising system.

Cyclic nucleoside monophosphates were suggested as possible prebiotic compounds (36,37), the driving force for polymerization being their high reactivity and the large negative standard enthalpy of hydrolysis. The prebiotic relevance of these polymerizations was questioned because efficient synthesis was observed with 2',3'- but not with 3',5'-cyclic forms.

In the possibly simplest activation system so far described, the phosphorylation of nucleosides by free phosphates or phosphate minerals in formamide was observed (5). The system affords 2'AMP, 3'AMP, 5'AMP, 2',3'-cAMP and 3',5'AMP providing prebiotically plausible precursors to polymerization.

Nucleoside phosphorylation also occurs in water (8). Treatment of adenosine in water with 1M KH$_2$PO$_4$ afforded the five phosphorylated forms. A high concentration of phosphate donor is necessary and in optimized conditions (16 hrs, 1M KH$_2$PO$_4$, 90°C, pH 6.1) the total amount of phosphorylated products reaches only the 7.3% of the input adenosine. In these conditions the half-lives of the open phosphorylated forms 2'AMP, 3'AMP and 5'AMP are 15, 23, and 35 hours, respectively, while the 2',3'- and 3',5'-cAMP cyclic forms have half-lives of 165 and 450 hours, respectively (11). Adenosine half-life in the same environments is 450 hours. Thus, although not efficiently and at high temperature, the formation of cyclic nucleotides also occurs in water.

Cyclic monophosphate nucleosides can be synthesized abiotically by a two-stage nucleobase assembly process on a sugar-phosphate scaffold, as shown for cytidine-2',3'-cyclic phosphate (38).

The stability of cyclic monophosphate nucleosides and of their precursor is of concern when one attempts to retrace the route followed by initial nascent ribopolymers. A possible solution is provided by the observation that in monophosphate ribonucleotides the 3' phosphate bond, the weakest bond in water, is stabilized upon polymerization (11). This property may endow the polymer with an evolutionary edge over the monomer, allowing accumulation of complex chemical information.

Protective conditions like inclusion in micelles, interaction with mineral surface (13) or inner strata, [i.e., in clays (39,40)], cycles of displacement into cooler surroundings, etc., might have played an important role in the formation and accumulation of activated precursors.

**On the mechanism of polymerization.**

The observed polymerizations only occur with cyclic nucleotides and do not take place with non-cyclic forms. Sizeable polymerization is observed only with 3'-5' cyclic nucleotides while the 2',3' cyclic ones only afford very short chains (up to tetramers).

These facts help to focus on the possible mechanism, based on the formation of the internucleotide bonds requiring the opening of the cyclic phosphate bridge. The nonenzymatic joining of oligoadenylates on a polyuridylic acid template was reported (37). In that case 3',5'-linked hexadecyl acid with a 2',3'-cyclic phosphate terminus was shown to couple on a polyuridylic acid template in the presence of ethylenediamine, yielding mostly a dodecamer.

Before that, syntheses of oligomers were obtained from 2',3'-cyclic AMP (36) upon polymerization on a poly(U) or from 2',3'-cyclic AMP evaporated from solution in the presence of catalysts such as aliphatic diamines (41). The self-polymerization afforded oligonucleotides of chain length up to at least 6. In both the reported reactions the opening of the phosphate cyclic bridge supposedly provided the necessary activation energy.

Nonenzymatic template-directed ligation of terminally pre-activated oligonucleotides was reported (19,20,23,42,43 and refs therein). In these works the formation of the internal phosphodiester bond is attributed to the template-mediated proximity of the reactive groups. At the contrary of these systems, the syntheses reported here require no special preactivation, no catalyst and no dry-chemistry, polymerization spontaneously occurring in water.

**A role for stacking interactions.**

The observed polymerizations occur in solution. The question thus arises as to how nucleic bases interact, rapidly and not based on sequence complementarity, and pertains in the first place to the conditions allowing stacking of nucleoside monophosphates in solution.

Stacking free energy profiles for all 16 natural ribonucleoside monophosphates in aqueous solution were reported (44-46). The potential of mean force calculations showed that the free energy profiles displayed the deepest minima and the highest barriers, and therefore the
highest stacking abilities, for the purine-purine dimers, especially for ApA and GpG. The free energy of stabilizing the stacked state were 2-6 Kcal/mol higher for purine-purine dimers than for pyrimidine-pyrimidine dimers. Base combinations with different stacking potentials (ApA > GpG > UpU ≃ CpC) (45) show a corresponding order of decreasing polymerization rate (A > G > U ≃ C), reinforcing the explanation that the formation of oligonucleotides in solution relies on stacking for the passage from monomer to short oligonucleotides.

The explanation for the formation of long sequences by terminal ligation [(8) and Figs 4 and 5] relies in the studies by Holcomb and Tinoco (47) and by Brahms et al. (48) who first described the double-strand formation by ribo PolyA and the relationship (48) between PolyA length and strand coupling. PolyA strands are held together by stacking (47,49), the double strands are parallel and ligate terminally (10) in the absence of enzymes, affording molecules with standard 3',5' bonds in their entire length (10). The stacking-unstacking process is considered to be temperature (46), pH (46,47) and fragment size (48) dependent, being in general favored by lower temperature and pH, and by longer size. The study of the free energy profiles of stacking for all 16 natural ribonucleosides monophosphates based on potential of mean force calculations shows that many different conformations, with different degrees of stacking are possible, revealing the gradual nature of the stacking phenomenon (45). This explains the variation in equilibrium constants and fraction stacked of ribonucleoside monophosphates reported (50-54) and predicts that various degrees of stacking may occur also in sub-optimal conditions, as higher temperature.

Hence, we hypothesize that the oligomerization reactions from 3',5'-cGMP and from 3',5'-cAMP described in Figs 1 and 2 rely on the stacking interaction of the purine moieties of the cyclic nucleotides, followed by the opening of the phosphodiester cyclic bond and the consequent formation of the inter-nucleotide phosphodiester bridge. This latter part of the reaction is favoured by high temperature.

The ligation process involved in the formation of the long A-stretches was described (10). The sequence extension due to the terminal ligation reaction reaction of PolyG on PolyC described in Figs 4 and 5 needs not to be different from this type of ligation. Nevertheless, while the PolyA ligation occurred on parallel-bound double-strands of A residues held by stacking, the latter occurred on antiparallel hydrogen-bonded base-paired double strands.

The versatility of the set of nonenzymatic polymerization reactions leading to longer sequences (Fig. 9) is possibly the most relevant property of these self-polymerizing systems.

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Table 1. Quantitative analysis of chain extension and terminal ligation test-systems results

| Polymer | Substrate/cofactor | Chain extension rate (a) | Half max (b) | Terminal Ligation rate (c) | Half max |
|---------|--------------------|--------------------------|--------------|---------------------------|----------|
| C<sub>24</sub> | 3',5'-cAMP | 0 | NA | 0 | NA |
| | 3',5'-cGMP | 4.600 ± 900 | 260 µM | Not determined (d) | NA |
| A<sub>12</sub>C<sub>12</sub> | 3',5'-cAMP | 0 | NA | 27 ± 10 | 2 µM |
| | 3',5'-cGMP | 10.000 ± 1.500 | 120 µM | 500 ± 100 | 2 µM |
| A<sub>24</sub> | 3',5'-cAMP | 0 | NA | 10.000 ± 1.500 | 2 µM |
| | 3',5'-cGMP | 0 | NA | 1.200 ± 200 | 2 µM |

(a) The chain extension rates were determined based upon densitometry measurements of autoradiograms of gel electrophoretic analysis of extension reactions (i.e., as in Fig. 4) and have been normalized with respect to the extension rate of 5’A<sub>12</sub>C<sub>12</sub>3’ in the best observed conditions (6 hrs, 60°C, pH 6.2, 1mM, 3’,5’-cGMP), which has been scaled to 10.000.

(b) Half max indicates the concentration of cyclic nucleotide at which the rate of product yield is one-half of the maximum extension or ligation rate.

(c) The terminal ligation rates were determined with the methodology described in (a), relative to the ligation rate of 5’A<sub>24</sub>3’ which has been scaled to 10.000 [see (8)].

(d) Oligo C does not dimerize. In the presence of 3’,5’-cGMP it forms a multimeric form due to a more complex phenomenon (as described in the legend to Fig. 4, panel A).

Cyclic pyrimidine nucleosides 3’,5’-cCMP and 3’,5’-cUMP do not promote chain extension nor ligation (Ref. 8, and this study; Fig. 4A, and data not shown). NA: not applicable.

**FIGURE LEGENDS**

**Figure 1. Nonenzymatic polymerization of 3’,5’-cGMP in water.**
Panel A: 3’,5’-cGMP reacted at 0.1 (lane 1), 1 (lane 2), 10 (lane 3) or 100 (lane 4) mM concentration at 85°C for 1 h in Tris HCl-buffered water, pH 8.2. *Navg* 10.32, 12.43, 10.19 and 8.88, respectively. Marker: hydrolyzed 24mer polyG, 16% polyacrylamide electrophoresis.
Panel B: Nonenzymatic polymerization of 3’,5’-cGMP at 60 (lane 1), 75 (lane 2) or 85°C (lane 3) for 1 hour in Tris-buffered water, pH 8.2, 1 mM 3’,5’-cGMP. The *Navg* after 1 h was 10.10, 11.08 and 11.84, respectively. The more represented species is the 8mer.
Panel C: 100 mM 3’,5’-cGMP. The *Navg* of the polymers obtained are 8.72, 9.02 and 9.34, respectively.
Figure 2. Non enzymatic polymerization of 3',5'-cAMP in water.
3',5'-cAMP reacted in water (85°C) for 30 min (lane 2) or 3 hours (lane 3). Lane 1: underexposure of part of lane 2. Marker: hydrolyzed 24mer polyA. Lane 4: blow-up analysis of the population of fragments encompassed between 20 and >120 nucleotides, 2x10^2 hours reaction, 12% acrylamide. RNA fragments obtained by ligation (10) were used as markers for lane 4 (not shown).

Figure 3. A simple model for the polymerization of 3',5'-cGMP.
The cyclic bond preferentially opens affording a 5’GMP (16). The guanine moieties of two of these molecules are supposedly held in position by stacking (44-46). Transfer of the bond is favoured at moderately high temperature. The 3’ phosphate bond is more stable in the polymer than in the monomer (11) thus justifying polymer prevalence in time.

Figure 4. RNA chain extension by 3',5'-cGMP-fed polymerization.
Panel A: 5’ labelled 5’C243’ reacted with 3’,5’-cAMP (lanes 3-5), 3’,5’-cGMP (lanes 6-8), 3’,5’-cCMP (lanes 9-11), or 3’,5’-cUMP (lanes 12-14). Each group of three lanes contained 0.1, 1.0 or 10 mM nucleotide, respectively. The reaction was in Tris-HCl-buffered water (pH 5.4) at 60°C for 6 hrs. Lane 1: U, untreated. Lane 2: no nucleotide. Insert: 1/3 autoradiographic exposure of the corresponding part of the gel. The model interprets the structure of the polymer in denaturing (left) and water condition (right). The polymer indicated by the asterisk is formed only in the presence of 3’,5’-cGMP. Its size is 84±3 nucleotides (as determined by band counting in the appropriate autoradiogram exposure and plot graphical extrapolation). This multimer is interpreted as a dimeric form of the extended monomer, possibly caused by Oligo G – Oligo G ligation. Oligo C- Oligo C dimerization does not occur in the absence of G-based cofactors or G extensions. The band corresponding to the 23mer is missing. For an explanation of the reduced hydrolysis of the last phosphodiester bond at the 3’ extremity, see (10). The 23mer is produced in enzymatic degradations (see Fig. 6).
Panel B: RNA chain extension by 3’, 5’-cGMP as a function of concentration. The reaction was performed as above in the presence of the indicated concentration of 3’,5’-cGMP.
Panel C: 5’ labelled 5’A12C123’ reacted with 3’,5’-cGMP. Lane 1: untreated. Lane 2: 6 hrs, Tris-HCl buffered water, pH 5.4, 60°C. The C12 segment undergoes hydrolysis faster than the A12 segment [as detailed in (17)]. Lanes 3-5: as in lane 2, in the presence of 0.1, 0.4 and 2 mM 3’,5’-cGMP respectively. The fragment sizes of the G-encompassing fragments were determined by top-down band-counting starting from the 48 mer dimer (24 x 2) in overexposed gel images.
Panel D: chain extension of 5’C243’ and of a 5’A12C123’ as a function of the 3’,5’-cGMP concentration. The data points show the % of full sized monomer molecules (filled symbols ▲ = 5’C243’, ● = 5’A12C123’) and of the extended molecules (open symbols, same) as a function of the 3’,5’-cGMP concentration indicated in abscissa. Data from experiments reported in panel B, and not shown).

Figure 5. The 5’A12C123’ oligo (schematically shown in its 3’ extremity - left) ligates to a neo-synthesized base-paired Oligo G segment through a standard 3’-5’ phosphodiester bond.
**Figure 6.** The chain extension reaction by 3',5'-cGMP on Oligo C is concentration-dependent.
The reaction was performed in the same conditions described in Fig. 4, in the presence of 10, 30, 60 or 90 μM 3',5'-cGMP for 0, 1, 10 or 60 min, as indicated. For each group of time points the amount of elongated chain does not vary.

**Figure 7.** T1 and SVPD I ribonuclease treatment of the 5’A₁₂C₁₂ G₈₋₇₅₃’ RNA, synthesized as in Figure 4 panel C.
Panel A: treatment of the 5’ labelled RNA with 40 units of T1 for the indicated times in Tris HCl-buffered water pH 7.2, 2 mM EDTA, in the presence of 1mM 3',5'-cGMP. Panel B: 400 Units. Units defined as in (12). The presence of 3',5'-cGMP markedly decreases the ribonuclease activity, as reported (55). Panel C: SVPD I, treatment as indicated in the figure, and as detailed in (10).

**Figure 8.** The half-life of an 5’A₂₄₃’ oligomer in the absence (“−”, left) or in the presence (“+”, right) of 20mM 3’,5’-cAMP. The oligomer was terminally labelled at 5’ and treated in water (90°C, pH 5.4) for the time indicated on top of each lane. Further details in (10, 17). The t ½ values observed are 36 hrs in the absence and 184 hrs in the presence of the cyclic nucleotide. Higher concentrations of the cyclic nucleotide further increased the t ½ values. U = untreated.

**Figure 9.** Abiotically formed cyclic precursors may actually have started their evolution towards complexity in a warm little pond, as first conceived by Darwin (9).
Figure 1C
Figure 2
$N_{\text{avg}} = 32.75$

Figure 4C
Figure 5
Figure 7
Figure 8
Generation of long RNA chains in water
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