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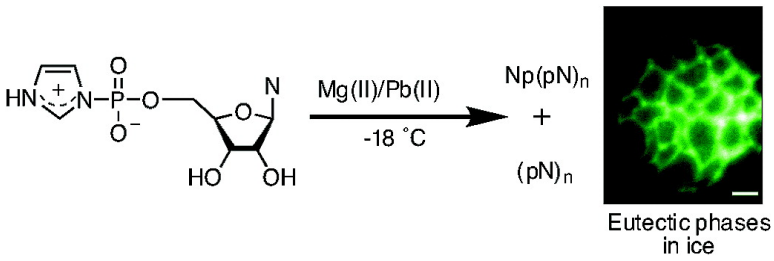

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Eutectic Phase Polymerization of Activated Ribonucleotide Mixtures Yields Quasi-Equimolar Incorporation of Purine and Pyrimidine Nucleobases

Pierre-Alain Monnard,* Anastassia Kanavarioti, and David W. Deamer

Contribution from the Department of Chemistry and Biochemistry, University of California, 1156 High Street, Santa Cruz, California 95064

Received June 2, 2003; E-mail: monnard@molbio.mgh.harvard.edu

Abstract: The RNA world hypothesis requires a plausible mechanism by which RNA itself (or precursor RNA-like polymers) can be synthesized nonenzymatically from the corresponding building blocks. Simulation experiments have exploited chemically reactive mononucleotides as monomers. Solutions of such monomers in the prebiotic environment were likely to be very dilute, but in experimental simulations of polymerization reactions dilute solutions of activated mononucleotides in the millimolar range hydrolyze extensively, and only trace amounts of dimers and trimers are formed. We report here that random medium-size RNA analogues with mixed sequences (5- to 17-mers with traces of longer products) can be synthesized in ice eutectic phases that are produced when dilute solutions of activated monomers and catalysts (Mg(II) and Pb(II)) are frozen and maintained at −18 °C for periods up to 38 days. Under these conditions, the monomers are concentrated as eutectics in an ice matrix. Hydrolysis of the activated mononucleotides was suppressed at low-temperature ranges, and polymerization was enhanced with yields up to 90%. Analysis of the mixed oligomers established that incorporation of both purine and pyrimidine bases proceeded at comparable rates and yields. These results suggest that ice deposits on the early Earth could have facilitated the synthesis of short- and medium-size random sequence RNA analogues and thereby provided a microenvironment suitable for the formation of biopolymers or their precursors.

Introduction

The discovery of catalytic RNA led to the proposal that an "RNA World" preceded the DNA−RNA−protein system that defines contemporary organisms.[1] The RNA world hypothesis posed an inescapable question: What synthetic mechanism could produce RNA molecules of sufficient length (up to 100-mer) to provide folded conformations required for ribozyme activity?[2,3] The problem may be divided into synthesis of medium-size (5- to 25-mer) and longer oligomers (25- to 100-mer). Earlier studies dealt with the oligomerization of monomers and dimers into oligomers up to 25-mer in length. For synthesis of longer oligomers, only elongation of preformed strands[4] or ligation reactions[5,6] can be envisioned.

It is reasonable to make a distinction between synthesis of medium-size and longer oligomers, such as ribozymes, in that the latter were likely to be products of an evolutionary process involving selection and directed synthesis.[7] A population of short- to medium-size oligomers composed of all the statistically plausible structures would be a good starting point for a pool that evolved via catalyzed ligation or elongation to longer RNA molecules with catalytic activity. In this context we have been looking for a microenvironment that is conducive to nonselective polymerization (phase I).

Condensation reactions required for phosphodiester bond formation are not favored in dilute aqueous environments.[8,9] To circumvent this difficulty in aqueous model systems, reaction mixtures containing metal catalysts and high concentrations of activated monomers, such as nucleotide imidazolides, have been used.[10,11] However, prebiotic oligomerization under these conditions is implausible due to limited availability of starting material[12] and low yields.[13] Alternative strategies have therefore been developed that utilize selective adsorption to mineral surfaces (for review see ref 9). For instance, if a purine imidazolide such as adenosine 5′-phosphoimidazole, ImpA, (14.5 mM) is incubated with montmorillonite clay for 3 days, oligomers up to 10 nucleotides in length are produced.[14] In the absence of clay catalysts only dimers and pyrophosphate dimers

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Table 1. Oligomerization Yields as a Function of Mixture Composition

composition (ImpN)	relative initial concentration U:A:C:G	total oligomer yield (%)[a,b]	trimers and longer oligomers (%)[b]
U, A, C, G	1:1:1:1	85.5 ± 2.5	52.1 ± 1.4
	4:4:1:1	88.4 ± 0.5	54.6 ± 1.5
	1:1:4:4	81.5 ± 0.8	48.0 ± 4.2
	16:1:1:1	89.0 ± 0.5	N. D.
U	1:0:0:0	87.8 ± 0.8	67.1[c]
A, C, G	0:1:1:1	84.1 ± 1.2	46.0 ± 2.2
U, A, G	1:1:0:1	85.3 ± 0.8	55.8 ± 1.3
U, A, C	1:1:1:0	83.4 ± 0.4	48.2 ± 3.8
U, C, G	1:0:1:1	81.4 ± 2.5	49.2 ± 4.4

[a] These percentages include dimers and longer oligomers detected in reaction mixtures (6 batches each) after 27 days at −18 °C. [b] The absorbance from which these percentages were calculated is not corrected for hypochromicity and is based on the initial total monomer absorbance. [c] The percentage was calculated using data from ref 18.

form. If a "primer" such as $(pdA)_9pA$ is included in the starting mixture, chain elongation with ImpA produces polymers ranging up to 50 nucleotides,[4] a length that corresponds to the minimal requirement for ribozyme activity.[15] Despite its success in producing long oligomers of RNA, this system is limited by inefficient incorporation of pyrimidine nucleotides.[16,17]

We have recently reported a nonenzymatic synthesis of polyuridylates (up to 11-mer) in frozen samples of phosphoimidazole-activated derivatives of uracil in the presence of Mg(II) and Pb(II) ions supplied as nitrate salts.[18] Polymerization most likely occurs in cavities between ice crystals that are filled with liquid eutectics of concentrated solutes and not by adsorption to ice crystal surfaces. Under these conditions, monomers and catalysts are concentrated to such an extent that up to 94% of phosphoimidazolide-activated uridylate, ImpU, is incorporated into oligomers. The polymerization of other activated nucleobases also took place forming homopolymers, with typical yields of ∼80% and lengths ranging up to pentamers.[18] In the present study, we report that the eutectic phases promote the synthesis of medium-size polyribonucleotides from mixtures of phosphoimidazolide-activated ribonucleotide monomers, ImpN's. This medium promotes heteropolymerization more efficiently than individual self-polymerization, allowing for the quasi-equimolar, quantitative incorporation of the four nucleotides into oligomers.

Results

Product Yields. We first investigated whether mixtures of activated monoribonucleotides could form products with yields equivalent to those obtained earlier during self-condensation experiments.[18] Table 1 summarizes the results obtained with ImpN mixtures using low initial concentrations of activated monomers (5 mM) and catalysts (5.2 mM Mg^{2+}, 0.6 mM Pb^{2+}).

All of the mixtures yielded high percentages of monomer incorporation, but mixtures with a relative excess of C/G nucleobases exhibited a 5−10% less efficient incorporation into polymeric material. Furthermore, on average, 46% (C-, G-rich mixtures, see Table 1) to 56% (U-, A-rich mixtures) of the starting material was detected in trimers or longer oligomers (in the elution region after $(pU)_3$, the first trimer to elute). Experiments with identical samples conducted in an unfrozen state (4 °C) yielded less than 20% total incorporation into oligomers up to 4-mer. These results were in good agreement with those previously obtained during self-condensation experiments.[18]

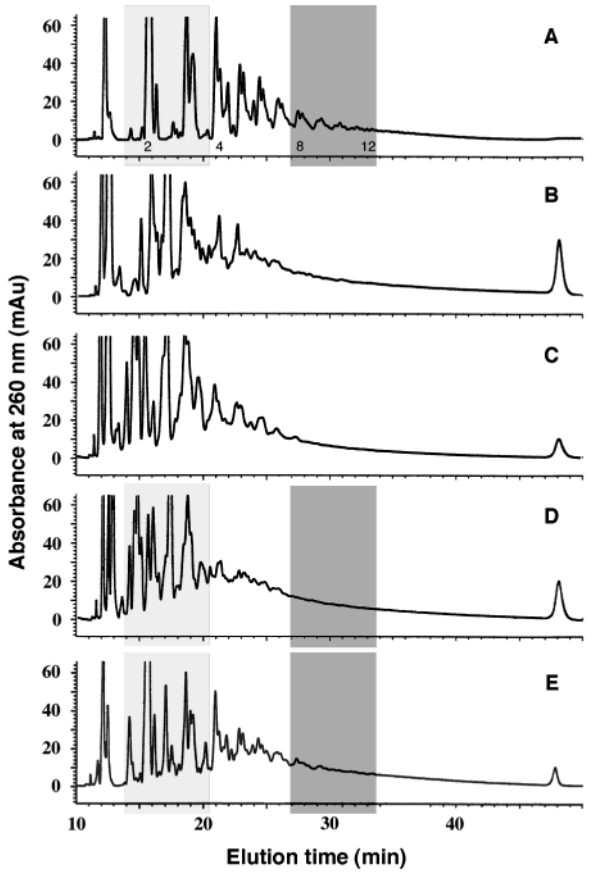

Figure 1. Product distribution in reaction mixtures with one, three, and four nucleobases. The samples were incubated for 27 days at −18 °C in the presence of both Mg(II) and Pb(II) ions. The total nucleobase concentration in every sample was 5 mM. HPLC profile (A) of products from the oligomerization of ImpU (U oligomer length is indicated), (B) of products from a ImpU:ImpA:ImpG oligomerization (1:1:1), (C) of products from a ImpA:ImpC:ImpG oligomerization (1:1:1), (D) of products from a ImpU:ImpA:ImpC:ImpG oligomerization (1:1:1:1), and (E) (ImpU:ImpA:ImpC:ImpG, 16:1:1:1).

As shown in Figure 1, the oligomerization of a single monomer (ImpU, Figure 1A) yielded products that were clearly separated by RPC-5/HPLC chromatography according to length. This reaction produced oligomers up to at least 13-mer, and the presence of even longer oligomers was indicated by a continuing UV absorbance that approached 0 only after 40 min elution time. In mixtures containing three or four nucleobases, the product distribution changed radically: In reaction mixtures containing three different nucleobases, new peaks appeared in the dimer to tetramer region (15−25 min elution), and peaks corresponding to 5-mer or longer oligomers tended to disappear progressively, replaced by a continuous line (Figure 1, parts B and C). This peak-pattern change was expected because the number of products synthesized from mixtures of monomers should increase exponentially with increasing oligomer length. Considering only the 3′-5′-linked oligomers formed from a mixture containing three monomers (and neglecting products

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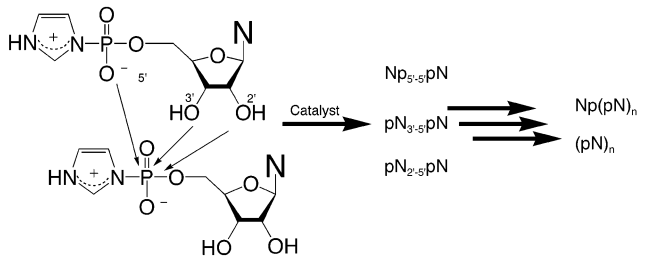

Figure 2. Reactivity of phosphoimidazole-activated ribonucleotide monomers (ImpN). Three different acyclic products can be formed during the condensation of two ImpN molecules: pyrophosphate (5′-5′), 2′-5′-, and 3′-5′-linked dinucleotides.

with 2′-5′, pyrophosphate capping and mixed linkages which are also yielded; see Figure 2), one calculates that 3^6 (729) different hexamers are possible products. HPLC/RPC5 chromatography does not have the resolution required to separate such a large number of sequences. This tendency was also observed in the HPLC profiles of products from mixtures containing all four nucleotides (Figure 1D).

The mixtures (Figure 1, parts B−D) also presented an additional peak at later elution times (48 min) corresponding to the wash cycle, which was usually absent in the polymerized ImpU, and represented up to 4% of the total monomer concentration. This peak presented a typical UV absorbance of nucleic acids and indicated the presence of long oligomers that were not eluted by the standard gradient (0−0.04 M $NaClO_4$ in 40 min). A steeper gradient (0−0.0335 M $NaClO_4$ in 31 min, then 0.0335−0.1 M in 22 min) eluted these products as a single broad peak before the wash, but without improving their resolution (data not shown). It is possible that stable oligomer aggregates might form by partial base pairing and then be retained longer on the HPLC column to appear as peaks. However, this seems unlikely since $(pG)_{11}$, which tends to form the strongest self-structures among oligomers, synthesized on poly(C) templates in ice eutectic phases can be quantitatively chromatographed under these conditions (data not shown). Such mixtures (four monomers at equimolar concentrations) produced a slow, but steady, increase in longer oligomers which eluted with retention times comparable to those observed for uridine homopolymers longer than 14-mer or adenine homopolymers longer than 10-mer.

Mixtures containing equimolar concentrations of the four nucleobases were also analyzed by gel electrophoresis (Figure 3). The gel lanes A2 and A3 clearly show blurred bands with partial length resolution (see arrows), which corresponded to polyribonucleotides at least as long as 17-mer with traces of longer polymers. The main products were 6- to 9-mers and 11- to 15-mers (Figure 3B, lane 2). A blurred band was expected because of the large number of possible sequences. In comparison, self-condensation of ImpU (Figure 3, parts A and B, Lane 4) yielded products that could be resolved by length up to 20-mer with traces of longer oligomers even though multiple isomers were present in the oligomeric material (see below).

In all mixtures, the length of the longest oligomers seemed to be conserved (HPLC data). This finding is interesting because in our previous report on homopolymer synthesis in eutectic phases, oligomers synthesized from activated A, C, and G nucleobases tended to be shorter than those observed with U self-polymerization.[18] Here, as the comparison with decomposed

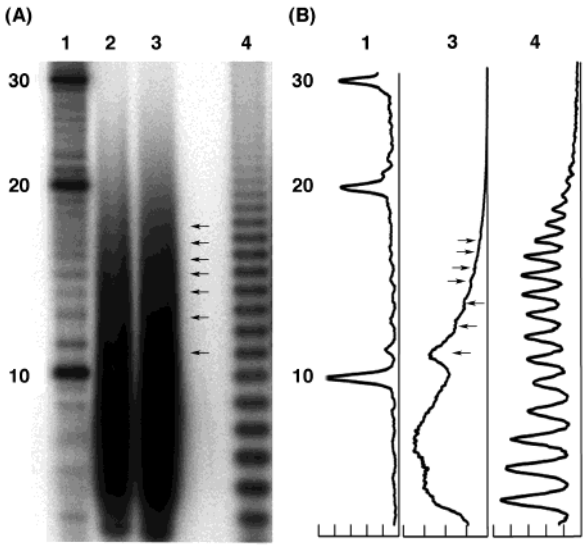

Figure 3. Determination of the polyribonucleotide length. (A) 20% polyacrylamide sequencing gel. Lane 1: Decade Marker System (Ambion). Lanes 2 and 3: oligomeric material formed in an equimolar four-nucleobase mixture after a 30-day incubation at −18 °C, 6 μL, and 10 μL of the same labeling reaction, respectively. Lane 4: a control ImpU self-condensation after a 30-day incubation at −18 °C. (B) Trace analysis of the gel. The maximum intensity was normalized for each lane. The lane numbers correspond to those in A. The arrows point to faint bands in the blurred lanes to suggest products partially resolved by length.

homopolymers established, the reaction mixtures (three and four nucleobases) all formed medium-size oligomers.

In reaction mixtures containing all four nucleotides, the product identity depended on the relative concentrations of each monomer. In Figure 1, yields of such mixtures (parts D and E) that contained large concentrations of ImpU were compared with the yield of a pure ImpU reaction (part A). The ImpU profile shows that after a 27-day incubation at −18 °C, oligomers at least 13 monomeric units long were produced with a total incorporation of 88%. The second mixture (Figure 1E) containing approximately 84 mol % of ImpU and 5 mol % of every other nucleobase illustrates the characteristic elution pattern changes observed with HPLC/RPC5 systems: Only peaks up to the heptamer are identifiable (Figure 1, dark highlighted profile portion). However, the monomer incorporation is similar to that previously observed (89%). This observation is important because these two mixtures contain comparable concentrations of ImpU, yet the small proportion of new nucleobases has markedly altered the product distribution of medium-length oligomers. As the ImpU molar excess decreased (Figure 1, parts D and E), the peaks tended to broaden and collapsed into a single peak. This would be expected if new mixed oligomers were gradually formed.

The total yield of oligonucleotides in the reaction mixtures containing three nucleobases did not suggest any preferential incorporation of purine over pyrimidine, as observed with methods relying on the adsorption of monomers on mineral surfaces.[16] Mixtures containing 2:1 or 1:2 ratios of purine to pyrimidine yielded approximately 81 to 85% of the initial monomer concentrations as oligomers. Therefore, eutectic conditions allowed both purine and pyrimidine nucleotides to be incorporated into oligomers with approximately equal efficiency.

Determination of the Nucleobase Content of the Polynucleotides. To investigate the mixed oligomer composition,

Table 2. Nucleobase Content of Oligomers (≥3-mer) Established by Phosphodiesterase Digestion

sample	relative initial concentration U:A:C:G	nucleobase incorporated into oligomers/total nucleobase present in whole reaction mixture				ratio of purine to pyrimidine
		U	A	C	G	
A	1:1:1:1	1.03 ± 0.01	1.34 ± 0.12	0.83 ± 0.03	1.06 ± 0.01	1.29
B	4:4:1:1	0.98 ± 0.03	1.13 ± 0.01	0.82 ± 0.06	1.06 ± 0.01	1.22
C	1:1:4:4	1.11 ± 0.15	1.53 ± 0.08	0.84 ± 0.02	1.13 ± 0.04	1.36

Table 3. Regioselectivity of Collected Long Oligomers (≥3-mer)

composition (ImpN)	relative initial concentration U:A:C:G	oligomeric material containing one or more 3′-5′ linkage (% of collected oligomers)	monomer incorporated in RNA analogues (≥3-mer) with at least one 3′-5′ linkage (% of the initial monomers)[a]
U, A, C, G	1:1:1:1	43.2 ± 4.0	22
	4:4:1:1	40.0 ± 5.0	21
	1:1:4:4	46.9 ± 10.2	23
U	1:0:0:0	30 ± 5[b]	20
A, C, G	0:1:1:1	48.7 ± 8.6	22
U, A, G	1:1:0:1	39.7 ± 16.9	22
U, A, C	1:1:1:0	37.2 ± 8.6	18
U, C, G	1:0:1:1	41.5 ± 6.6	20

[a] This percentage corresponds to oligomer yields (%, Table 1, fourth column) multiplied by oligomeric material containing one or more 3′-5′ linkage (%, Table 3, third column). [b] This percentage includes the linkage present in the dimeric product (18).

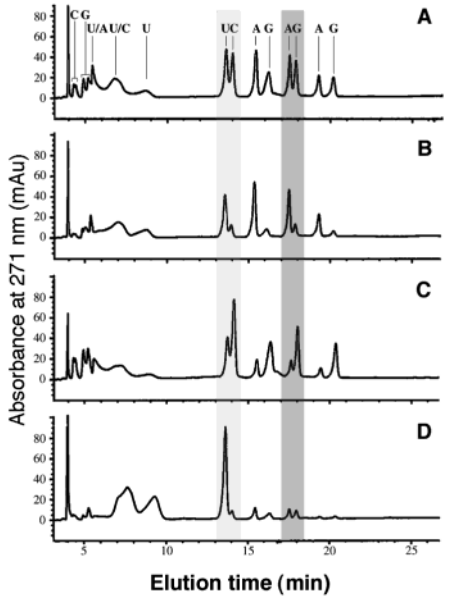

Figure 4. Identification of the nucleobase content in the polyribonucleotides. Trimers and longer oligomers were collected and hydrolyzed under alkaline conditions. The analysis was performed by HPLC/C-18 chromatography, and the peaks were assigned to each nucleobase by co-injecting standards or UV analysis of their chromatogram. Nucleobase composition of the oligomeric material formed in an equimolar four-nucleobase mixture (ImpU, ImpA, ImpC, ImpG, 1:1:1:1) (A), in a mixture with an excess of A/U (ImpU, ImpA, ImpC, ImpG, 4:4:1:1) (B), in a mixture with an excess of C/G (ImpU, ImpA, ImpC, ImpG, 1:1:4:4) (C), and in a mixture with an excess of U (ImpU, ImpA, ImpC, ImpG, 16:1:1:1) (D).

the nucleobase content of oligomers longer than dimers was carried out by alkaline or enzymatic degradation (phosphodiesterase). These procedures ensured the total hydrolysis of oligomeric products, although partial deamination of cytidine (yielding uridine) occurs at high pH used in the alkaline degradation. The samples were then analyzed by HPLC/C-18 chromatography.

As shown in Figure 4, every nucleobase that was present in the starting reaction mixture appeared in the HPLC/C-18 profiles of hydrolyzed nucleic acids, indicating its incorporation in oligomeric material. Moreover, the absolute nucleobase content varied according to the initial concentration of the activated monomers. When the two highlighted portions of the HPLC profile at 271 nm are considered, which correspond to U/C (light highlight, Figure 4) and A/G peaks (dark highlight, Figure 4), it is obvious that the initial differences in monomer content (Figure 4B, U:A:C:G; 4:4:1:1 and 4C, U:A:C:G, 1:1:4:4) were reflected in the absolute nucleobase content of the oligomeric material.

The nucleobase distribution in phosphodiesterase-digested oligomer (≥3-mer) fractions was compared with that in similarly treated reaction mixtures which contained the hydrolyzed and still-activated monomers, dimers, and oligomers (see Table 2). In these fractions, oligoribonucleotides were slightly depleted in cytidine, enriched in adenine, and contained a proportion of uridine and guanosine comparable to that in the whole reaction mixture. The ice eutectic synthetic conditions produced almost equimolar incorporation of each monomer. For an initial U:A:C:G 1:1:1:1 monomer mixture, the incorporation ratio was on average 1.24:1.61:1.00:1.28, or 1.29 purines for each pyrimidine. This value confirmed that there was little preference for incorporation of purines over pyrimidines.

Comparable relative nucleobase incorporations were observed in every digested sample, which also suggests that the effect of ice eutectic phases was not related to a specific nucleobase mixture, but applies for a wide range of conditions.

Determination of the Regioselectivity. The regioselectivity in the mixed ribonucleotide oligomerization (percentage of 3′-5′ linkages) was determined by enzymatic digestion with RNase ONE which cleaves 3′-5′ linkages exclusively, leaving 2′-5′- or pyrophosphate-linked RNA fragments intact. Samples subjected to enzymatic digestion and samples incubated for the same time period in the absence of RNase ONE were analyzed with HPLC/RPC5 chromatography. The percentage of 3′-5′ linkages in the oligomeric material (≥3-mer of uridine) was calculated based on the degradation pattern (Table 3).

In all mixtures, oligomers with one or more 3′-5′ linkages amounted to approximately 40% of the total oligomeric material, in contrast to previous values (≤10%) found in solution experiments with lead and magnesium as catalysts.[19,20] A similar

percentage was found in dimeric products (5′-purine−pyrimidine, 5′-pyrimidine−purine, and 5′-purine−purine) on montmorillonite.[17] However, the marked decrease in 3′-5′ linkages in 5′-pyrimidine−pyrimidine reported for clay-catalyzed reactions was not observed in the oligomeric products of mixtures with a ratio of pyrimidine to purine of 2:1 formed in ice eutectic phases. It is interesting that U:A:C:G mixtures with an excess of G:C nucleobases yielded lower total incorporation (1:1:4:4 and 0:1:1:1) and produced higher 3′-5′ linkage content (46.9 and 48.7%).

In general, the eutectic conditions modified the ratio of 2′-5′ to 3′-5′ linkages in favor of the natural isomers, in mixed-monomer polymerization even more than in self-polymerization. This trend was previously observed in solution experiments with binary and tertiary mixtures at high concentrations of monomers.[21] Finally, the incorporation of monomers into oligomers (≥3-mer) with at least one 3′-5′ linkage (approximately 22% of the initial activated monomer pool; Table 3) was comparable for all mixtures tested here.

Oligomer Sensitivity to RNase A and RNase T1. Trimers and longer oligomers were isolated from the reaction mixtures and subjected to enzymatic degradation by RNase T1 and RNase A. Degradation of the oligomeric material (≥3mers) resulting in a range of monomeric and dimeric products would further substantiate the mixed character of polymer sequences yielded in eutectic phases in ice. RNase A is a pyrimidine-specific nuclease that degrades natural C−N (N = A, C, U, or G) or U−N linkages whereas RNase T1 cuts 3′ of guanosine residues indicating the presence of a natural G−N linkage. Because of the presence of polyribonucleotides containing unnatural linkages (2′-5′ and pyrophosphate), the enzymatic degradation only offered qualitative information.

In all of the reaction mixtures containing three activated nucleobases (U:C:G, A:C:G, and U:A:G), every sample incubated in the presence of either enzyme exhibited degradation patterns (appearance of monomers and dimers) that were more extensive than those observed in control samples. This indicated that the polyribonucleotides were composed of mixed sequences rather than single nucleobase stretches.

Discussion

It is possible that a variety of ice matrixes would have been available on the prebiotic Earth. An extreme case of global ice was proposed by Bada et al.,[22] who suggested that low temperatures would tend to preserve organic compounds and thereby make them available for chemical evolution. In the work presented here, we have demonstrated that in the presence of transient ice matrixes, eutectic phases within the matrix are conducive to nonenzymatic polymerization of activated ribonucleotides at low initial concentrations of both the activated monomers and metal catalysts.

A wide range of mixtures of activated monoribonucleotides yielded comparable amounts of medium-length RNA analogues after a relatively short incubation period at −18 °C. Gel electrophoresis established the presence of oligomers at least 17 units long, with traces of longer products.

Polyribonucleotides of mixed sequences were synthesized even in mixtures containing high pyrimidine concentrations. This conclusion is supported by the overall yields (which were comparable in all reaction mixtures; Table 1), the separation patterns resulting from RPC-5 HPLC and gel electrophoresis, and the sensitivity of oligomeric products in three-nucleobase mixtures to specific RNases (RNase A and RNase T1). The self-condensation of ImpG and ImpC in eutectic phases in ice yielded fewer oligomeric products of shorter length than the ImpU self-condensation,[18] but mixtures containing an excess of these two nucleobases did not noticeably inhibit the condensation reaction.

The high yields supported by eutectic phases in ice presumably are the result of concentrating effects. However, simply increasing the concentration of activated ribonucleotide monomers up to 1.35 M in three nucleotide mixtures at 20 °C only slightly increases the yields of medium-length oligomers.[21,23] Furthermore, in eutectic phase reactions, an 8-fold variation of the initial monomer concentration had little effect on the oligomer length in ImpU self-condensation.[18] This suggests that several additional properties of eutectic phases may also contribute, such as preservation of the activated species and polymers, more stable base stacking, and perhaps ordered monomer assemblies on the ice crystal surface.

In terms of regioselectivity, the oligomeric material contained approximately 40% product with one or more 3′-5′ linkages (Table 3), a percentage comparable to that observed with clays.[17] This yield is higher than that observed in solution[11,20] or by self-polymerization reactions in ice eutectic phases.[18] Even mixtures initially containing 66% pyrimidine nucleobases produced a large proportion of 3′-5′-linked oligomers, a result not observed on montmorillonite.[17]

The results reported here also establish that oligomers (≥3-mer) formed in eutectic phases contain each nucleobase in approximate proportion to its initial concentration in the reaction mixture. The more efficient condensation of purines reported previously with clay[17] or in dilute solutions[21] was not observed here. On average, for each pyrimidine, 1.2−1.3 purines are incorporated in the eutectics (Table 2). Also, the reactivity sequence determined on montmorillonite (A > G > C > U)[17] or in solution with or without template (purine > C > U)[24] does not apply to the eutectic phases in ice where the reactivity sequence is A > G ≈ U > C. However, the comparable total incorporation yields and the nucleobase distribution in the oligomers suggest that the small reactivity differences between activated monomers do not strongly affect the formation of longer oligomers even in mixtures containing excess cytidine.

Prebiotic Relevance of a Nonenzymatic Polymerization in Eutectic Phases in Ice. The lack of high-yield prebiotic reactions[25] leading to synthesis of biochemically relevant building blocks suggests that solutions of organic compounds on the early Earth were likely to have been highly dilute. For polymer synthesis to occur, concentrating mechanisms relatively independent of dilution would be required. Prebiotic simulations of polynucleotide synthesis generally use temperatures in the range 0−25 °C, even though subzero temperatures are better at preserving activated monomers and oligonucleotide prod-

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ucts.[22,26,27] Eutectic phases in ice provide both concentrating power and monomer and oligomeric product preservation for an efficient nonenzymatic polymerization.

RNA analogue polymerization in eutectic phases rapidly forms large quantities of medium-length polymers, which could be used as a template or primer for further elongation. For instance, oligomers longer than 5-mer are required for efficient adsorption and elongation catalyzed by clay surfaces.[4,28,29] Mixed oligomers, if reactivated by a yet unknown mechanism, would also lead to the formation of even longer strands by ligation events and perhaps templating.[30] In this sense, an efficient nonenzymatic synthesis of medium-length mixed oligomers, such as those produced in ice eutectics, may fill a gap in providing a population of oligonucleotides containing all four bases that could have led to a postulated RNA world.[1]

The efficient pyrimidine incorporation reported here is also essential for information transfer in an RNA-based genetic system. If RNA fragments were relevant to the emergence of life as both catalyst and information repository, a set of four monomers would be required to avoid the severe information limitation imposed by a two-base system consisting of purine nucleotides. Even in a three-nucleobase scenario, the need for an efficient incorporation of at least one pyrimidine nucleobase is obvious. One such genetic code is based on the assumption that the wobble G·U base pair would be substituted for the Watson−Crick G·C base pair, since cytidine is the least stable of the four extant nucleobases.[31] This system might be sufficiently complex to form secondary and tertiary structures required for an active ribozyme. As demonstrated by Joyce and co-workers,[32] the cytidine content of certain ribozymes can be chemically altered (C bases become U) without extensive loss of activity.

The almost quantitative incorporation of U derivatives into the oligomers obtained in eutectic phases in ice is also interesting because substituted uracils have been proposed to act as a scaffold for peptide synthesis and thereby bridge an RNA world with the DNA−protein world.[33] For these reasons, the reactivity sequence in eutectic phases in ice (A > G ≈ U > C) would be advantageous.

Our working hypothesis is that the eutectic phases in ice concentrate, order, and preserve the activated nucleobases and products, allowing for a much more effective polymerization to take place. This implies that any other solute present in the initial reaction mixture will also be concentrated in the eutectic phases in ice, for example, the MES buffer and the nitrate counterions in our reactions. On the early Earth, inorganic ions, such as Na^+, Ca^{2+}, Fe^{2+}, Cl^-, and SO_4^{2-}, were likely present at various concentrations depending on the aqueous environments (thermal vents, ocean, streams, and ponds on land masses). These ions would have been concentrated in the eutectic phases and in initially high ionic solutions would have inhibited to some extent the polymerization described here but also other processes essential for the emergence of life, such as membrane self-assembly.[34]

Conclusion

Within the limitations of our model system (use of imidazole-activated monomers, presence of mixed linkages), the eutectic phases in ice seem to be conducive to a truly random RNA analogue polymerization, which could yield pools of medium-size polymers. In a second phase, these RNA analogues could selectively elongate or ligate to form oligomers in the range of 100 nucleotides. These two phases are essential for the development and evolution of a metabolism proposed in the RNA world scenario.[1]

Materials and Methods

Reagent-grade materials were used throughout, and solvents were HPLC grade. The reagents were purchased from the following sources: 2(*N*-morpholino)ethane sulfonic acid (MES), Sigma; magnesium nitrate hexahydrate >99.5% and lead(II) nitrate 99%, Fluka; [γ-^{32}P] ATP, Amersham Pharmacia; alkaline phosphatase with a specific activity of 20 u/μL, Roche Applied Sciences; T4 polynucleotide kinase with a specific activity of 10 u/μL, NEN; Ribonuclease ONE (RNase ONE) with a typical activity of 10 u/μL, Promega; Phosphodiesterase I from *Crotalus atrox*, Ribonuclease A (RNase A), and Ribonuclease T1 (RNase T1) with a typical activity of 0.01 u/mg, 90 Kunitz u/mg, and 322 u/μL, respectively, Sigma. HPLC columns were packed by hand with RPC-5 solid phase, kindly provided by L. E. Orgel (Salk Institute, San Diego, CA).

The sodium salts of nucleoside 5′-phosphoimidazolides, ImpN (*N* = Adenosine, Cytidine, Guanosine, Uridine; see structure in Figure 2), were prepared in 98 ± 1% purity as described in ref 11. Samples were always prepared from freshly made stock solutions of the substrates to preserve their purity.

Sample Preparation. The samples were prepared from stock solutions of 50 mM ImpN solutions except for ImpG (12.5 mM), 30 mM $Pb(NO_3)_2$, 60 mM $Mg(NO_3)_2$, and 50 mM MES, pH 6.5. The final concentrations were achieved by diluting the stock solutions with Millipore water. A typical sample with a 600 μL volume contained 5 mM total monomers, 5.2 mM Mg(II), 0.6 mM Pb(II), and 5 mM MES buffer, pH 6.5. The pH of all reaction mixtures was in the range of 6.6−6.9 at room temperature and remained stable during incubation. The samples were frozen at −18 °C in a cooling bath (Polyscience) and then maintained at that temperature for periods up to 38 days. Higher temperatures did not result in complete freezing of the samples. The reaction mixtures sometimes contained precipitates, but no correlations between precipitates and low yields could be established. In previous experiments, we established that $SnCl_2$, as a solid, catalyzed the reaction as well.[18]

Detection of Oligomer Formation. Products were separated on an RPC-5 HPLC column as described previously.[18] Aliquots of reaction mixtures were quenched with EDTA (final concentration 8.7 mM) and maintained at +10 °C in the HPLC's autosampler before analysis. The products were eluted with a linear aqueous $NaClO_4$ gradient at pH 12 made with NaOH (no $NaClO_4$ for 4 min, then 0−0.04 M $NaClO_4$ in 40 min at 1 mL/min). Product yield is expressed as the sum of the HPLC units reported under the oligomeric product peaks over the sum of the HPLC units reported for all the peaks (monomer and oligomers) that corresponds to the initial amount of substrate monomer present. Only samples with the same (±5%) total HPLC areas were compared. Oligomer yields were not corrected for hypochromicity and are therefore slightly underestimated (approximately 10%).

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The length of the oligomeric products was estimated by comparison of the retention times of products from partial degradation of poly(U) and poly(A) chromatographed under the same conditions and by co-injection of pU3′pU with poly(U) to determine the retention time of the dimers. Alternatively, the samples were dialyzed against Millipore water using DispoDialyzer (MWCO 500) from Spectrum Laboratories, Inc. to remove the catalyst, imidazole, and buffer molecules. Polyribonucleotides were then subjected to alkaline phosphate (removal of the 5′ phosphate) and end-labeled (10 μCi [γ-^{32}P] ATP for 15 μL reaction mixture diluted twice) with T4 polynucleotide kinase (10 units per labeling reaction). Radioactive products were fractionated by gel-electrophoresis (20% polyacrylamide), exposed on image plates (Molecular Dynamics), and analyzed using ImageQuant (Molecular Dynamics).

To purify the oligomers, the samples were eluted at 1 mL/min on HPLC/RPC5 chromatography using the following gradient: no $NaClO_4$ for 4 min, then 0−0.010 M $NaClO_4$ in 8 min, isocratic for 3 min, and 0.010−0.150 M in 1 min. This particular gradient eluted all oligomers longer than $(pU)_3$ as a single peak. The eluate was then dialyzed against Millipore water using micropore dialysis bags (MWCO 500) from Spectrum Laboratories, Inc. to remove perchlorate salt.

To detect oligomer degradation products, reverse-phase HPLC chromatography was used with a 1090 LC system (Hewlett-Packard) equipped with a diode array detector and an Alltima C-18 column, 4 × 250 mm, 5 μm from Alltech with a guard (C-18). The analysis was performed at pH 2.5: solvent A, 0.02 M KH_2PO_4 with 0.2% (w/v) trifluoroacetic acid (TFA), pH 2.15; solvent B, 30% CH_3CN in water (v/v) with 0.2% (w/v) TFA; 0−15% B in 10 min, an isocratic step followed (4 min at 15% B); 15−60% B in 45 min, and 60−100% B in 2 min. The flow was 0.5 mL/min.

Nonenzymatic and Enzymatic Degradation of the Oligomers. Collected oligomeric material was decomposed by alkaline hydrolysis (1 M NaOH, 24 h at 39 °C), and base composition was determined by HPLC/C-18 chromatography, using co-injected samples and standards or comparing UV/vis spectra obtained on the diode array detector.

In some experiments, nucleobase content of oligomers was determined by enzymatic digestion with phosphodiesterase, an enzyme that cleaves all phosphodiester bonds (1.5 u/mL, pH 8.8, 37 °C for 2 days). To establish the ratio of unnatural to natural linkages in the collected oligomers, reaction mixtures were digested by RNase ONE (20 units per 25 μL reaction mixture, pH 7.0, for 2.5 days at 37 °C) which exclusively cleaves 3′-5′ linkages of linear RNA fragments between any two ribonucleotides. Samples incubated at 37 °C without enzyme and after enzymatic digestion were analyzed with HPLC/RPC5 chromatography. On the basis of the degradation pattern, the percentage of 3′-5′ linkages in the product could be calculated.

To confirm synthesis of mixed oligomers, samples containing three nucleobases were digested by RNase A, an endonuclease that cleaves the phosphodiester bond between the 5′-ribose of a nucleotide and the phosphate group attached to the 3′-ribose of an adjacent pyrimidine nucleotide. Samples (25 μL) were mixed with 10 μL 200 mM Tris, 40 mM EDTA, pH 7.5, and 5 μL 18 Kunitz units/μL RNase A, then incubated for 1 day at 37 °C. In other experiments, RNase T1 was employed, which specifically cleaves RNA at the 3′ end of guanosine residues. In this case, 25 μL samples were mixed with 10 μL 200 mM Tris, 40 mM EDTA, pH 7.5, and 5 μL 18 Kunitz units/μL RNase T1, followed by incubation for 1 day at 37 °C. The activity of both enzymes was checked by comparing samples incubated similarly in the presence/absence of enzymes.

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