

Synthesis of Pyrimidines and Triazines in Ice: Implications for the Prebiotic Chemistry of Nucleobases

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Abstract: Herein, we report the efficient synthesis of RNA bases and functionalized *s*-triazines from 0.1 M urea solutions in water after subjection to freeze–thaw cycles for three weeks. The icy solution was under a reductive, methane-based atmosphere, which was subjected to spark discharges as an energy source for the first 72 h of the experiment. Analysis of the products indicates the synthesis of the *s*-triazines cyanuric acid, ammeline, ammelide, and melamine, the pyrimidines cyto-

sine, uracil, and 2,4-diaminopyrimidine, and the purine adenine. An experiment performed as a control at room temperature, with the urea solution in the liquid phase and with the same atmosphere and energy source, led to the synthesis of hydantoins and insoluble tholin, but there was no evidence of

the synthesis of pyrimidines or triazines. The synthesis of pyrimidines from urea is possible under a methane/nitrogen atmosphere only at low temperature, in the solid phase. The generation of both pyrimidines and triazines in comparable yields from urea, together with a possible role for triazines as alternative nucleobases, opens new perspectives on the prebiotic chemistry of informational polymers.

Keywords: heterocycles • nucleobases • origin of life • prebiotic chemistry • triazines

Introduction

The prebiotic synthesis of nucleic acid bases is a central issue in the RNA-world hypothesis, one of the main proposals for the origin of life, based on the self-assembly of nucleic acid monomers. Possible scenarios for the synthesis of nucleic acids are still under debate, and despite the abiotic synthesis of several nucleobases, the relevance of these syntheses to the origin of life is not well established.

In a pioneering work, Ferris et al. obtained cytosine from urea and cyanoacetylene and from sodium cyanate solution

and cyanoacetylene.^[1] The isolation of the latter from spark discharges in methane/nitrogen mixtures suggests the relevance of this synthesis to the origin of life.^[1,2] Cyanoacetylene is present in the atmosphere of Titan, in comets, and in the interstellar medium and, thus, is indeed of prebiotic relevance.^[3] A quarter of a century later, Robertson and Miller reported the synthesis of cytosine in high yields (30–50%) upon heating various concentrations of urea and cyanoacetaldehyde in a sealed ampoule.^[4]

Recently, the prebiotic relevance of pyrimidine synthesis from urea and cyanoacetaldehyde was discussed.^[5,6] The main concerns are the availability and instability of the reactants. In the case of cyanoacetaldehyde, it could 1) react with amino acids, 2) undergo hydrolysis to generate formate and acetonitrile, or 3) form a dimer. Consequently, any cyanoacetaldehyde is unlikely to survive long enough to be available in sufficient quantity to produce the necessary concentration for cytosine synthesis. On the other hand, urea decomposes to ammonia and carbon dioxide at pH < 5 and hydrolyses at pH > 5 with a gradual decrease in concentration.^[7] Urea could also react with amino acids to form *N*-carbamoyl amino acids or hydantoins.

A way to overcome these problems, along with the problem of cytosine instability through deamination to form uracil, has been hypothesized in which ice–water solutions

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are used to generate the appropriate conditions for the reaction through the exclusion of solutes from concentrated interstitial brines in the ice matrix. This exclusion leads to a concentration enhancement in the microenvironments in the ice that has been shown to be of up to six orders of magnitude upon slow freezing of aqueous solutions with an organic molecule like methylene blue.^[8] In addition, freezing extends the lifetime of labile molecules and gives time for them to be processed further, due not only to the protective effect of the ice but also to the low temperatures, which tend to retard reaction mechanisms that are prevalent in the liquid phase. Indeed, the unique environment generated in the interstitial liquid channels in ice, with high pressures and strong gradients, could favor certain reactions, such as nucleotide polymerization.^[9]

On the basis of this hypothesis, cytosine and uracil were synthesized from cyanoacetaldehyde and urea in a frozen solution at -11°C , the eutectic point of the urea/water system.^[10] However, the constraints on the prebiotic validity of a synthesis from two components, one of them very unstable, mean that the problem of the origin of pyrimidine nucleobases persists. Thus, an experimental prebiotic simulation that produces bases with a good yield and under a plausible combination of prebiotic components and energy is still necessary. When it is taken into account that no other synthesis of cyanoacetaldehyde besides that from cyanoacetylene has been demonstrated, along with the reported syntheses of cytosine from cyanoacetaldehyde and of cyanoacetylene from methane/nitrogen mixtures, it is theoretically possible to perform the synthesis of pyrimidines from a methane atmosphere.^[11] However, to date, this synthesis has not been described in the literature.

This work explores the prebiotic importance of the suggested cyanoacetylene/urea pathway to cytosine and offers an icy scenario for the synthesis of pyrimidines and triazines from methane/nitrogen and urea. We also suggest a model for the possible chemical pathways generated by spark discharges in methane/nitrogen atmospheres and how the presence or absence of icy surfaces, in conjunction with urea as a reactant, drives the course of the reaction through these proposed chemical routes.

Results

A freeze–thaw cycle (repetition of -5 to 5°C variations) was generated in a degassed, sterile 0.1 M urea solution (pH 7.1) under a nitrogen/hydrogen/methane (30:30:40) atmosphere by using a thermostatic reactor (Figure 1). This atmosphere is reducing in nature and constitutes the primary carbon source for the experiment.^[12] Once the freeze–thaw cycle was established in the entire solution pool, the system was energized by means of spark discharges during the solid phase of the cycle. A tungsten electrode connected to a high-frequency and high-voltage generator induced a discharge that impacted the water/ice surface. As a reference, the solution is fully frozen at -5°C . The colligatively de-

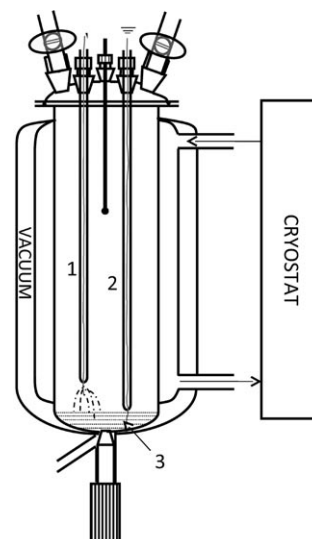


Figure 1. Illustration of the experimental system used in this study. A thermostated reactor equipped with an energy source was filled with the gas mixture used in the experiment and with the liquid urea solution. After sealing, the temperature cycles were established by using a programmable cryostat filled with silicon oil. 1) and 2) Tungsten electrodes connected to a high-voltage (50 KV) generator and to the ground, respectively; 3) urea solution in water.

pressed freezing temperature (estimated from the cryoscopic constant of H_2O , $\lambda = 1.86 \text{ K mol}^{-1}$) was $T_f \approx 273.15 - 1.86 \cdot [\text{urea}] = 272.96 \text{ K}$ for 0.1 M urea.

After 72 h, the voltage generator was disconnected, and the reactor was maintained with active freeze–thaw cycling. After 3 weeks, the reactor content, a yellowish solution with a pH value in the range 8.4–8.8 (3 experiments performed) and without any suspended organic solid, was allowed to warm to room temperature and was collected as fast as possible in sealed headspace vials under a nitrogen atmosphere to avoid excessive manipulation and to minimize the contamination risk. The solution was freeze dried, and the dry yellowish solid was analyzed.

The reaction product (Figure 2) contained unreacted urea and the triazines cyanuric acid (2,4,6-trihydroxy-*s*-triazine), ammelide (6-amino-2,4-dihydroxy-*s*-triazine), ammeline (4,6-diaminotriazin-2-ol), and melamine (2,4,6-triamino-*s*-triazine). These compounds were identified as tri(trimethylsilyl) (TMS) derivatives, by using the agreement of the retention times with those of standards as the criterion for identification, together with the mass spectra, by using the following ions for determination: m/z 345 and 330 for cyanuric acid; m/z 344, 329, and 171 for ammelide; m/z 343, 328, and 171 for ammeline; and m/z 342, 327, and 171 for melamine. The reaction also yielded the pyrimidines cytosine, uracil, and 2,4,5-trihoxypyrimidine. These pyrimidines, 2,4-diaminopyrimidine, and the purine adenine were identified as TMS derivatives by using the following ions for determination: m/z 254, 240, and 170 for cytosine; m/z 256 and 241 for uracil; m/z 279 and 264 for adenine; m/z 254 and 239 for 2,4-diaminopyrimidine; and m/z 344 and 329 for 2,4,6-

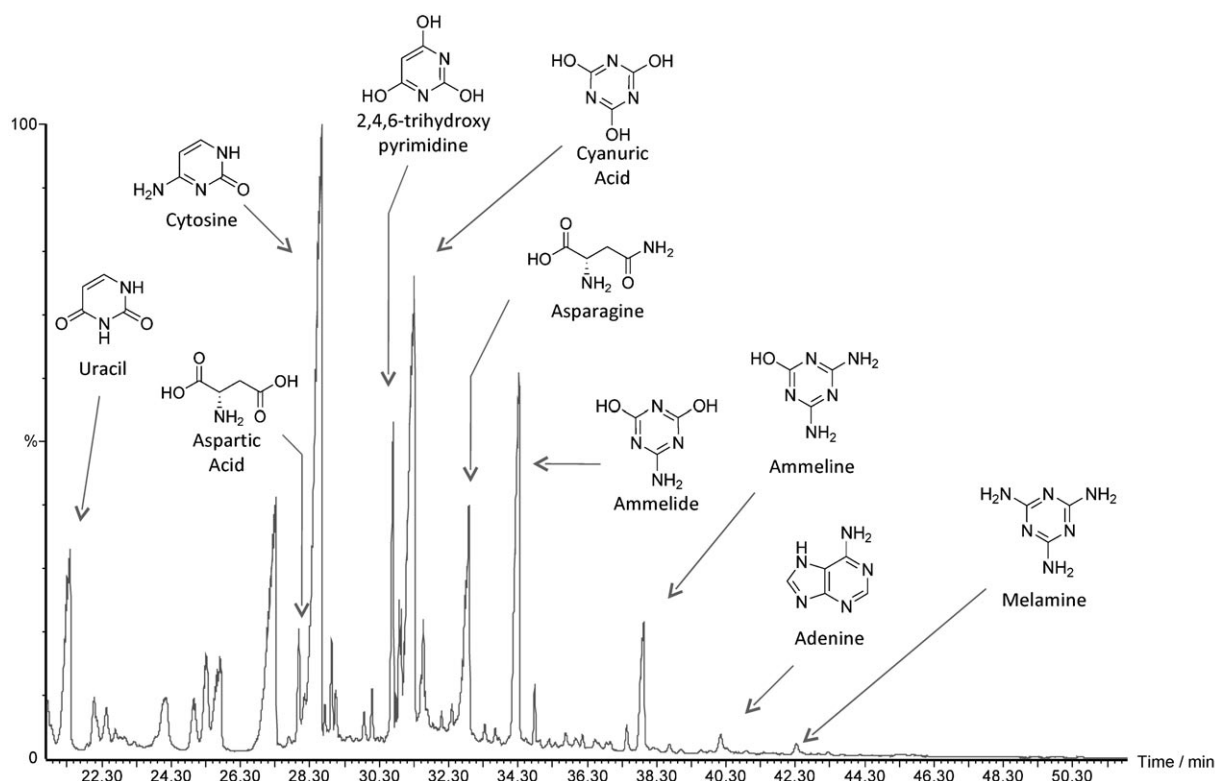


Figure 2. Gas chromatogram showing the trimethylsilyl derivatives of pyrimidines and triazines obtained from spark discharges between a $\text{CH}_4/\text{N}_2/\text{H}_2$ atmosphere and a 0.1 M urea solution subjected to freeze–thaw cycles. Residual urea (not shown) appears as a strong peak (urea–TMS derivative; m/z 204, 189, 147, and 73) at a retention time of 19 min.

trihydroxypyrimidine. The major products were cyanuric acid and cytosine. Overall, the ratio of cytosine/hydroxypyrimidines was 1 (Table 1). The only amino acids identified in the reaction product were glycine (not shown in the chromatogram), asparagine, and aspartic acid. The hydantoins, which were the major products in the control experiment (see below), were also present in minor quantities.

To test the effect of the freeze–thaw cycles on the chemistry of the system, one control experiment was performed at room temperature with the same experimental setup. Under

Table 1. Yields of triazines and nucleobases obtained by sparking of a 0.1 M urea solution subjected to freeze–thaw cycles under a $\text{CH}_4/\text{N}_2/\text{H}_2$ atmosphere (experiment 1), subjected to freeze–thaw cycles under an inert atmosphere (experiment 2), or under the atmosphere of experiment 1 with the urea solution maintained in the solid phase (experiment 3). Yields are calculated based on the total urea amount introduced into the experiment.

Compound	Experiment 1 Yield [%]	Experiment 2 Yield [%]	Experiment 3 Yield [%]
cyanuric acid	7.1	2.8	2.1
ammelide	2.8	1.6	0.8
ammeline	1	–	detected
melamine	0.02	0.1	–
cytosine	4.2	–	1.6
uracil	1.9	–	1
2,4,6-trihydroxypyrimidine	0.9	–	–
adenine	0.15	–	–

these conditions, the reaction product contained a significant quantity of insoluble organic material (32.5% of the carbon introduced as methane and urea in the experiment). The solution contained unreacted urea, glycine (not shown in the chromatogram), hydantoin, substituted hydantoins, parabanic acid, and carbamoyl-glycine (Figure 3). The pyrimidines and purines were undetected and the triazines were detected with low yields (<0.01%).

Due to the known behavior of urea in an aqueous solution subjected to a phase change, we took into account the possibility of the generation of triazines directly from urea by a mechanism of polymerization similar to those proposed for triazine generation from molten urea. A control experiment was performed by sparking the urea solution with the same conditions as the freeze–thaw cycles but under an inert atmosphere (argon). Under these conditions, we saw the formation of triazines but not pyrimidines (Figure 4). The yields of the triazines obtained in this experiment were significantly lower than those under a methane/nitrogen atmosphere. This could be due to argon ionization, which diverts the electric discharge in all directions around the electrode and diminishes the energy input into the ice–water pool. It is interesting to note that we found melamine but not ammeline under these conditions. Further study of the chemistry of urea solutions with energy sources and the effect of concentration and temperature on this is in progress in our laboratory.

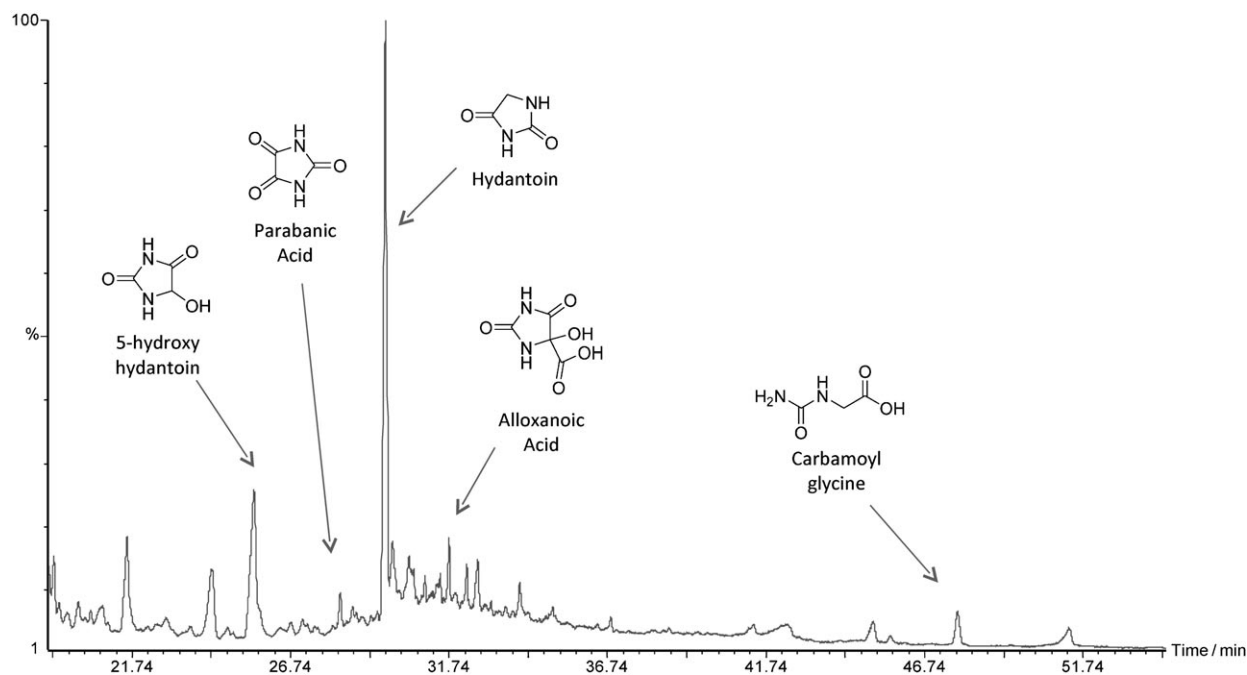


Figure 3. Gas chromatogram showing the trimethylsilyl derivatives of hydantoin derivatives obtained from spark discharges between a $\text{CH}_4/\text{N}_2/\text{H}_2$ atmosphere and a 0.1 M urea solution at room temperature. Residual urea (not shown) appears as a strong peak at a retention time of 19 min.

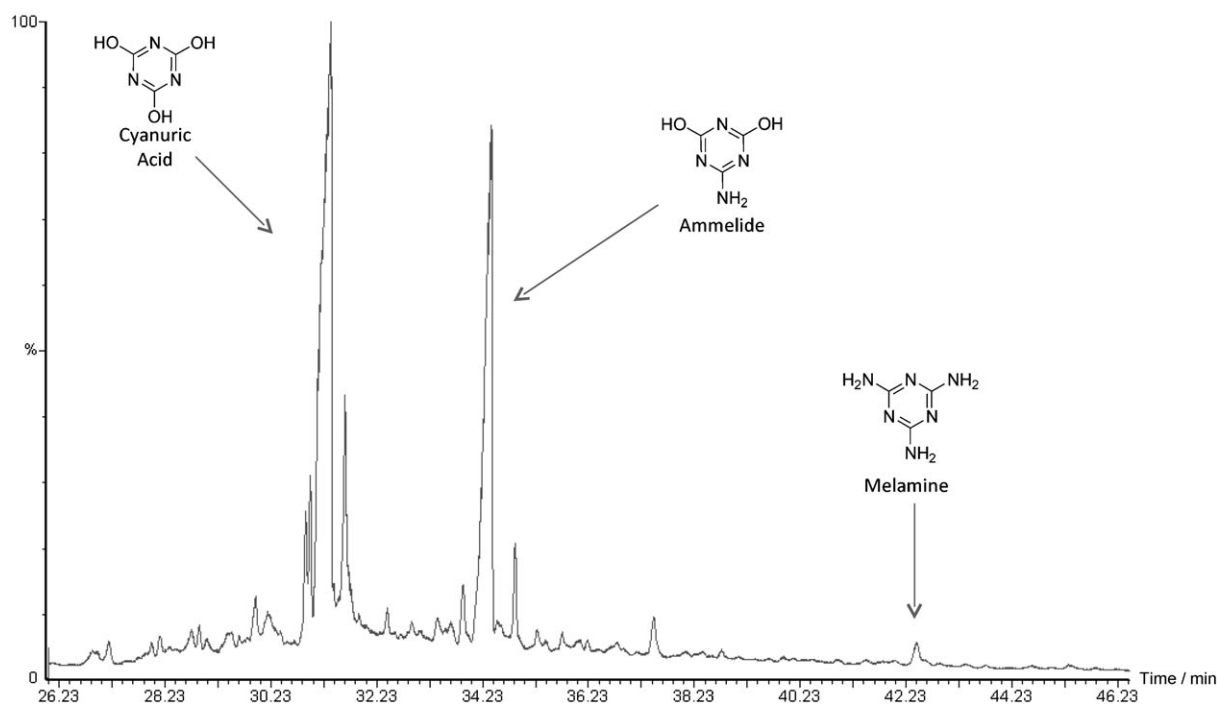


Figure 4. GC/MS chromatogram (total-ion-count mode) showing the trimethylsilyl derivatives of triazine derivatives obtained from a urea solution subjected to freeze–thaw cycles and spark discharges under an inert atmosphere (argon). Due to the lower yields obtained under these conditions, the residual urea appears as a strong peak that saturates the system. To avoid damage to the mass spectrometer, the ion registration begins at 26 min.

The effect of freeze–thaw cycles is a key phenomenon, as we showed by comparison with an experiment performed entirely at low temperature with water in the solid phase. Under these conditions, we found that mostly triazine deriv-

atives were formed. Also, we found that the concentration of pyrimidines and the overall yields were significantly lower than those found for the freeze–thaw-cycle experiments.

To test whether urea suffers degradation or biological contamination in solution when subjected to freeze–thaw cycles in our reactor design, a third control experiment was performed without spark discharges under the methane/nitrogen/hydrogen atmosphere. After three weeks of freeze–thaw cycles, the solution content was analyzed; no evidence of degradation, microbial contamination, or the generation of nitrogen heterocycles was found.

Discussion

Our experiments show that the synthesis of pyrimidines under a methane/nitrogen atmosphere is possible with high yields if a urea source is present. In this process, the presence of frozen water or ice is a decisive factor. With water subjected to freeze–thaw cycles, the synthesis of pyrimidines and triazines is strongly favored and the generation of insoluble or polymeric organic materials or tholins is negligible. Previously reported experiments on the synthesis of cytosine from cyanoacetaldehyde and guanidine or urea in ice at -11°C suggested a preference for uracil generation and the deamination of aminopyridines or their precursors during formation.^[10] Under our conditions, the observed preference sequence of pyrimidines was cytosine > uracil > 2,4-diaminopyrimidine > 2,4,6-trihydroxypyrimidine. Further work is in progress to establish whether long-term storage in ice under freeze–thaw cycles alters the concentrations of the resultant pyrimidines.

The behavior of urea in an aqueous solution subjected to a phase change could be a key factor in the results observed with water in different phases. The urea molecules in fluid-water solutions form pronounced hydrogen bonds with the neighboring water molecules at both the amino and the carbonyl groups.^[13] The number of hydration water molecules per molecule of urea has been reported as approximately 2 at concentrations of less than 5.0 M urea.^[14] Neutron diffraction measurements at 25°C on aqueous 15 mol% ($\approx 10\text{M}$) urea showed that approximately 4.3 water molecules are hydrogen bonded to the carbonyl oxygen atom.^[15] Infrared and dielectric spectroscopy studies showed that the two predominant interactions are those of urea–urea (observed at urea concentrations higher than 1 M) and water–urea. At a concentration of 11 M, nearly all of the urea molecules have other urea molecules as their nearest neighbors, because few water molecules remain to hydrate the urea molecules at high concentrations. Concentration-dependence studies showed that this is due to the aggregation of urea molecules in dimers and/or oligomers at higher concentrations.^[14,16] There is a considerable amount of urea dimer or clusters present in aqueous solutions.^[13]

Upon freezing of an aqueous solution of a polar organic compound, the solute is segregated from the pure ice to accumulate in supercooled microfluids. The solute retains a certain amount of water in the form of a supersaturated solution. Under these conditions, the system is governed by dehydration and the association of the solute molecules.^[17]

In analogy to another polar organic compound, such as pyruvic acid, in frozen aqueous solutions,^[18] we can expect that the urea remains monomeric in frozen aqueous urea solutions. The extent of urea dimerization ($\approx 18\%$ in 0.1 M urea at 25°C ^[19]) is expected to increase at lower temperatures and higher urea concentrations and to approach completion a few degrees below the onset of freezing.^[8,17] Thus, in our frozen-water experiment, urea is present as hydrogen-bonded urea dimers or higher association clusters and is randomly distributed within the ice. In this environment, water can still hydrate the polar organic molecules,^[17,18] but the urea–urea interactions will predominate.

Triazine derivatives are obtained in the laboratory and industry as products of the pyrolysis of urea.^[20] These compounds were found in the Murchinson and Orgueil meteorites, and it has been argued that their origin could be the hydrolysis of macromolecular material present in the meteorite^[21] or the polymerization of hydrogen cyanide,^[22] which could form the unsubstituted *s*-triazine. We have shown that the polymerization of HCN is not a necessary condition for the generation of triazines when the urea–water system is present at low temperatures (Figure 4). This implies that several alternative pathways could be active, dependent on the temperature and the presence of the reactants. As in the case of the synthesis of polycyclic aromatic hydrocarbons (PAHs)^[23] and as stated in the previous paragraph, we could expect that the ice medium, due to its surfaces and the freezing process, would favor a mechanism similar to those of the pyrolysis processes of urea, which could explain the synthesis of triazines.

In fact, the control experiment performed under an inert atmosphere shows the formation of triazines in the same relative abundance as the main experiment. During pyrolysis, part of the urea undergoes isomerization to ammonium isocyanate and decomposition, which releases ammonia. The reaction of the isocyanic acid, also released, with urea generates biuret (aminocarbonyl urea). The biuret reacts with more isocyanic acid and generates cyanuric acid and ammonia, ammelide, or ammeline. The latter could react with ammonia to form melamine. This reaction sequence, proposed for the pyrolysis of urea, is not observed by heating below the urea melting point and is consistent with the relative abundance of *s*-triazines found with our conditions: cyanuric acid > ammelide > ammeline > melamine.^[20]

The photodecomposition of urea under UV radiation is a possible source of isocyanic acid and ammonia as an alternative to thermal decomposition.^[24] Under our conditions, dissipation of spark energy in the form of ultraviolet radiation and the associated photochemical processes could be one of the sources of isocyanic acid, together with the local effect of the spark and the behavior of urea in frozen solutions. In fact, the fluid microenvironments or urea oligomers generated in the ice matrix could be comparable to the molten urea and could facilitate the condensation reactions.

With regard to the relevance of our results to prebiotic evolution, the importance of functionalized *s*-triazines would not be diminished, because they could act as purines

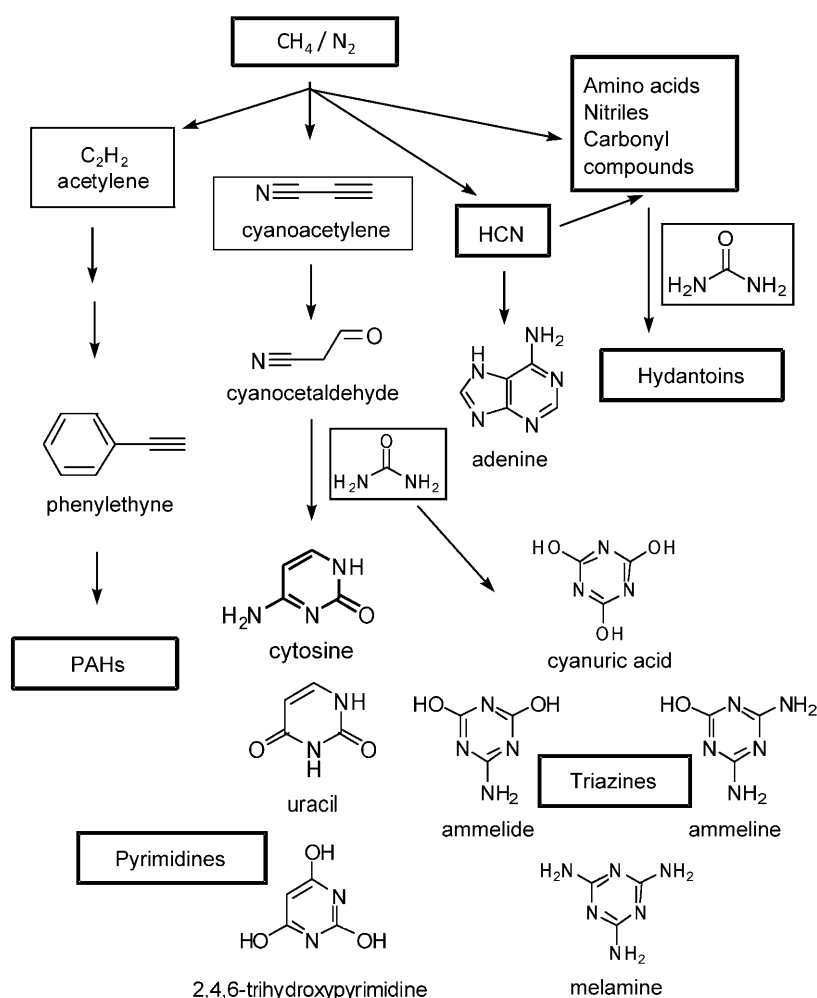
or pyrimidines in nucleic acid molecules,^[25] a role studied by Mittapalli et al.,^[26] who observed that oligopeptoids tagged with triazines as potential primordial informational polymers showed an imbalance of pairing behavior and weak base pairing. On the other hand, 2,4-diaminotriazines in peptidic oligomers establish strong base pairing with 2,4-dioxypyrimidines in nucleic acid backbones.^[26] We obtained pyrimidines and triazines as the main products and, taking into account the low yields of purine bases in the synthesis, we do not preclude the possibility of a primordial informational polymer containing triazines that was gradually rejected during evolution due to weak base pairing and instability issues.

The control experiment performed with water in the liquid phase at ambient temperature with the same atmosphere and urea concentration did not show the formation of either triazines or pyrimidines (Figure 3). The main organic material obtained in the liquid-phase experiment was insoluble tholin, with similar properties to the material obtained in previously reported experiments performed in our laboratory at room temperature.^[27] The soluble organic material obtained in the control experiment in the liquid phase contained mainly unreacted urea and hydantoins. The generation of hydantoins could be related to reactions between urea and amino acids, amino nitriles, or carbonyl compounds, generated in the spark experiment at room temperature.^[26,27] The Urech synthesis from α -amino acids and cyanate (which could be generated by urea isomerization) and the Simonson synthesis or Heintz synthesis from urea yield 5-functionalized hydantoins. The Bucherer–Bergs synthesis yields 5,5'-functionalized hydantoins.^[28]

We also identified carbamoyl-glycine at ambient temperature. This product, formed by the reaction of urea with glycine, appears to be absent in the experiment performed in ice. However, given that cyanoacetylene and hydrogen cyanide are the main products of spark discharges, we must expect the formation of aspara-

gine or aspartic acid under hydrolysis-favorable conditions, as a byproduct.^[5] We found both asparagine and aspartic acid, which suggests that, when a urea solution undergoes freeze–thaw cycles, the generation of pyrimidine nucleobases is the preferential reaction.

The urea solution subjected to freeze–thaw cycles favors the generation of nucleobases, diminishes the formation of insoluble tholin and other polymeric organic material, and reduces the importance of side reactions between urea and amino acids or their precursors generated in classic spark experiments. Moreover, the pyrimidines are more stable at low temperatures, and the ice matrix gives them a chance to undergo further chemical evolution to higher complexity. The microenvironments in which the molecules are located are surrounded by a medium that is more reflective and can



Scheme 1. Possible synthetic pathways in a urea solution subjected to freeze–thaw cycles under a methane/nitrogen atmosphere, with spark discharges between an electrode and the ice/liquid surface as an energy source. The pathways lead to those chemical families found in the reaction product: the synthesis of aromatics by the generation and polymerization of acetylene, the synthesis of pyrimidines from cyanoacetylene and urea, the synthesis of triazines from the possible isocyanic acid generated during urea decomposition, and the synthesis of hydantoins from urea and amino acids, cyanohydrins, hydroxy acids, or other carbonylic precursors. These suggested pathways are based on the final products obtained. The pathway leading to hydantoin synthesis and the generation of insoluble organic material (tholin) prevails in the liquid phase. By contrast, the frozen medium favors the generation of pyrimidines and triazines.

be protective towards photodimerization. It also provides higher stability against deamination due to lower temperatures, which prevent thermal decomposition within the time-scale of our experiment. Our results confirm the potential importance of ice in planetary environments, such as Mars, Saturn's icy moons, or the primitive Earth, as previously suggested.^[11]

In summary, the spark discharges in a methane/nitrogen atmosphere and freeze–thaw cycles operate by two basic mechanisms: first, the generation and polymerization of acetylene, which leads to aromatic compounds and acetylene-derived polymers, as previously reported in experiments with pure water under freeze–melt conditions,^[23] and second, the generation of cyanide derivatives, such as cyanoacetylene. Urea could act as a cyanoacetylene scavenger, favoring the mechanism of formation of nitrogen derivatives (Scheme 1). Therefore, the presence of urea reduces the availability of reactants for the generation of cyanide or acetylene polymers and explains the lack of insoluble organic materials obtained in experiments with urea/ice. At room temperature, the classic mechanism of atmospheric generation of tholins and amino acid precursors appears to prevail and leads to the formation of hydantoins without the formation of pyrimidines or triazines, despite the presence of excess urea in the solution.

Conclusions

The freezing process offers an interesting environment that favors the abiotic synthesis of molecules of biochemical interest and could be a favorable scenario for the laboratory exploration of prebiotic chemistry in planetary environments. The ice matrix plays the role of a protective medium that avoids the degradation of molecules such as the pyrimidines, enhances the yields, and diminishes the side reactions, which constitute the constraints for the actual prebiotic relevance of cyanoacetylene, acetylene, or urea.

The classical synthesis of cytosine and uracil from cyanoacetylene/cyanoacetaldehyde and urea could only be performed by using a urea solution under a methane/nitrogen atmosphere if the solution is subjected to freeze–thaw cycles.

The high-yield synthesis of triazines opens a way to explore these molecules as mimics of prebiological nucleobases in the early stages of evolution and could help to explain their presence in meteorites.

Experimental Section

Urea-solution freeze–thaw cycles: 0.1 M Urea solution (50 mL) in ultrapure, degassed water was frozen at -5°C in a sealed and thermostated glass reactor under an atmosphere of nitrogen (30%), hydrogen (30%), and methane (40%) at atmospheric pressure. The system was energized with a high-voltage generator (Model BD-50E, Electrotechnic Products Inc., IL, USA) by high-frequency spark discharges (50 KV, 0.5 MHz) directly into the water, through a tungsten electrode attached to the reactor

(electrically earthed with a secondary tungsten electrode). Freeze–thaw cycles were established by varying the temperature between -5°C and 5°C (2 h at -5°C , ramping to 5°C at a rate of $0.1^{\circ}\text{Cmin}^{-1}$, and 2 h at 5°C) with a Haake Phoenix II programmable cryostat (Thermo Electron Corporation). The system was maintained under spark discharge for 72 h. After this period, the high-voltage generator was disconnected and the reactor kept sealed with active freeze–melt cycles for three weeks. After that, the system was allowed to warm to room temperature, and the yellowish aqueous solution was stored in sealed headspace vials under an inert atmosphere.

For the control experiment with an inert atmosphere, argon of the maximum purity available (99.9995%) was used.

Gas chromatography–mass spectrometry: The solid, freeze-dried sample was derivatized with *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS; provided by Pierce, Rockford, IL, USA) according to this protocol: dried sample (1 mg) was combined with BSTFA/TMCS (0.1 mL) in a dry glass vial, and the mixture was stirred and heated at 60°C for 3 h. After that, a sample (1 μL) was transferred to the injection port of a GC apparatus in the splitless mode, and the analysis was performed by using an Elite-5 (Perkin–Elmer) 5% phenyl-95% methylsiloxane capillary column (30 m \times 0.25 mm; 0.25 μm film).

Mass spectrometry analysis was performed by using a Perkin–Elmer Autosystem XL-Turbomass Gold quadrupole apparatus in the EI+ scan mode. Organic compounds were identified by a search for their mass spectra in the NIST database, and the identified compounds were confirmed against authentic standards (provided by Sigma–Aldrich) by comparison of mass spectra and retention times. For identification purposes, we considered only peaks with a signal-to-noise ratio over 20. Those peaks for which the match probability in the database was below 90%, those that were matched but for which authentic samples were commercially unavailable, and those that were only tentatively identified were considered unidentified and are not discussed in this paper.

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