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To be cited as: *ChemBioChem* 10.1002/cbic.202000510

Link to VoR: https://doi.org/10.1002/cbic.202000510

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10.1002/cbic.202000510

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Prebiotic origin of pre-RNA building blocks in a urea "warm little pond" scenario.

Cesar Menor-Salván*[a,c] Marcos Bouza[a,b], David M. Fialho[a,b], Bradley T. Burcar[a,b] , Facundo M. Fernández[a,b], and Nicholas V. Hud[a,b]

[a] Prof. C.Menor-Salvan, Prof. N. V. Hud, Dr. M. Bouza, Dr. D. Fialho, Dr. B. Burcar, Prof. F. Fernández.
NSF-NASA Center for Chemical Evolution
Georgia Institute of Technology
30302 Atlanta (USA)
E-mail: cesar.menor@chemistry.gatech.edu, Nick.hud@chemistry.gatech.edu

[b] Prof. N. V. Hud, Dr. M. Bouza, Dr. D. Fialho, Dr. B. Burcar, Prof. F. Fernández
School of Chemistry and Biochemistry
Georgia Institute of Technology
30302 Atlanta (USA)

[c] Prof. C. Menor-Salvan
Dep. de Biología de Sistemas/IQAR
Universidad de Alcalá
28806 Madrid (Spain)

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Abstract: Urea appears to be a key intermediate of important prebiotic synthetic pathways. Concentrated pools of urea likely existed on the surface of the early Earth, as urea is synthesized in significant quantities from hydrogen cyanide or cyanamide (widely accepted prebiotic molecules), it has extremely high water solubility, and it can concentrate to form eutectics from aqueous solutions. The dual nucleophilic-electrophilic character of urea makes it an ideal precursor for the formation of nitrogenous bases. We propose a model for the origin of a variety of canonical and non-canonical nucleobases, including some known to form supramolecular assemblies that contain Watson-Crick-like base pairs. These reactions involve urea condensation with other prebiotic molecules (e.g., malonic acid) that could be driven by environmental cycles (e.g., freezing/thawing, drying/wetting). The resulting heterocycle assemblies are compatible with the formation of nucleosides and, possibly, the chemical evolution of molecular precursors to RNA. We show that urea eutectics at moderate temperature represent a robust prebiotic source of nitrogenous heterocycles. The simplicity of these pathways, and their independence from specific or rare geological events, support the idea of urea being of fundamental importance to the prebiotic chemistry that gave rise to life on Earth.

The *de novo* prebiotic formation of RNA during the process of life origination is challenging, due to the discussed availability or plausibility of some reagents, and the complexity of the numerous steps required for the synthesis of ribonucleotides and their subsequent polymerization. In recent years, the question of the formation of the canonical components of RNA received intensive research[1–5]. The seeking of routes to RNA from plausible abiotic precursors on early Earth is not exempt of problems[6], and that have led many scientists to view the RNA World as an intermediate stage in the origins of life, with simpler polymers serving in catalytic and informational roles before the emergence of RNA[7–10]. The possibility of pre-RNA structures and precursors widely opens the evolutionary perspective of the origin of life, as there exist alternative nucleobases that readily form nucleosides with ribose and other sugars[11,12], and the expanded space of structures is compatible with the view that the evolution of pre-biopolymers resulted in functionally superior RNA and DNA[8]. We propose that concentrated solutions of urea were essential for pre-RNA chemical evolution. This proposal is based upon two primary observations: (1) urea is produced in model prebiotic and astrochemical reactions from one-carbon sources, such as cyanide, cyanamide, methane, or carbon oxides[13–15], and (2) urea is relatively stable, allowing it to accumulate over time on the surface of the early Earth. Urea forms high-concentration viscous solutions[16] that favor its accumulation in evaporating ponds. The abundance of urea in the presence of other prebiotic components would allow for the creation of large-scale stable eutectic as low-water environments. In these environments, wet-dry cycles could have easily concentrated and selected molecules, promoting reactions potentially important for initiating life. The atmospheric formation of reactive species over a concentrated solution of urea (using methane or acetylene as precursors and UV radiation or spark discharges as energy sources) subjected to freeze-thaw cycles has been shown to result in the formation of pyrimidines, triazines, and purines; significant yields being observed of barbituric and cyanuric acid, together with their corresponding aminopyrimidines and aminotriazines[17,18]. Hence, the formation of eutectic urea solutions in a prebiotic environment could constitute a good scenario for the origination of nucleobases; this property could be added to its previously reported ability to promote phosphorylation of alcohols, including nucleosides[16], and its physicochemical properties that are potentially useful for pre-enzyme nucleic acid evolution[19]. Heterocycle synthesis in urea solutions is of renewed interest with the recent discovery of high-yielding model prebiotic reactions for the ribosylation of melamine, 2,4,6-triaminopyrimidine (TAP) and barbituric acid[11,20]. These nucleoside analogs are particularly interesting as they can form supramolecular assemblies as monomers with complementary heterocycles (e.g., TAP with cyanuric acid, melamine with barbituric acid). The *C*-riboside formed by barbituric acid can be regarded as a prebiotic analog of uridine and pseudouridine, which opens the possibility of pre-RNA, informational carrying structures with non-canonical nucleobases that are extremely

close to the extant nucleobases[20]. Taken together, these results suggest an early important role of urea as a precursor to pre-RNA and extant RNA building blocks[9]. We hypothesized that urea enriched ponds could be a source of non-canonical nucleosides, if malonic acid is present in the prebiotic chemical space. In preliminary results[21] we showed that malonic acid, which could be formed by hydrolysis of malononitrile[22,23], by irradiation of urea[13], or by prebiotic proto-metabolic cycles, as the malonate cycle[24], could condense efficiently with urea in a "warm little pond" model to yield pyrimidines in prebiotic conditions. In this communication we further explore the formation of nucleobases and non-canonical nucleosides of interest for chemical evolution[8], in a prebiotic *urea-warm little pond* using malonic acid as precursor.

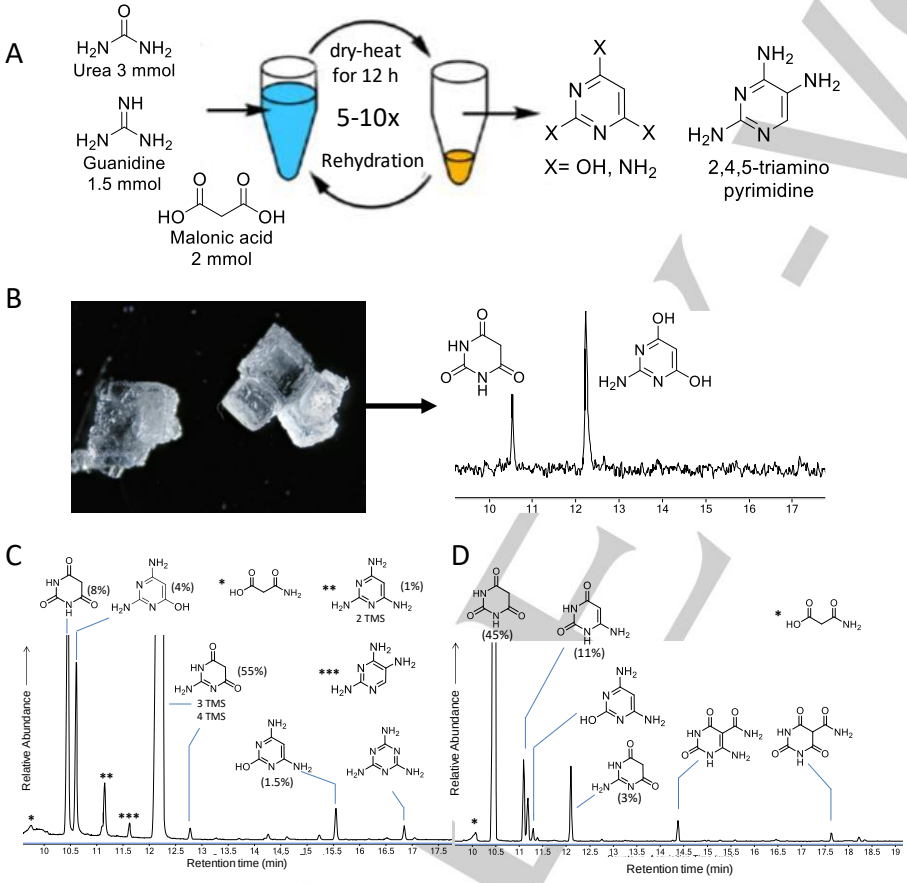


Figure 1. (A) Synthesis of 2,4,6-functionalized pyrimidines by condensation of urea/guanidine and malonic acid during environmental cycles under prebiotic conditions; (B) Cocrystals of barbituric acid and 2-aminopyrimidine-4,6-diol formed during dry/wet cycles experiment at 65ºC; (C) GC-MS chromatogram showing the trimethylsilyl derivatives of the products of condensation of malonic acid in an equimolar urea/guanidine solution subjected to cycles of drying and momentary rewetting. Product yield indicated at each structure; (D) GC-MS chromatogram showing the trimethylsilyl derivatives of the products resulting from the condensation of malonic acid and urea in a solution subjected to dry/wet cycles at 65ºC.

To explore whether the malonic acid-urea condensation could be a prebiotic reaction for the origin of the barbituric acid family of pyrimidines, we subjected a solution of 2 mmol urea and 0.5 mmol malonic acid to dry-wet cycles: 85ºC for 6 hours in the dry phase, followed by rehydration and heating at 60ºC for 12 hours in the wet phase. After 3 days cycling, barbituric acid crystals had grown. The solution was lyophilized, and the viscous residue analyzed after formation of trimethylsilyl derivatives (Fig S1 A). The analysis by gas chromatography-mass spectrometry (GC-MS) showed the formation of barbituric acid with a measured 42% yield with respect to initial malonic acid. Also, 2-amino-4,6-dihydroxypyrimidine and 6-aminouracil were found in low yields, as well as expected 5-carbamoylated products[25]. The dry/wet cycling of the urea-malonic acid solution did not reveal significant production of triazines.

If urea is replaced by biuret in the same conditions, cyanuric acid is formed (Fig. S1 B). These results are consistent with our previous results on the formation of s-triazines in an ice matrix[18], in which we postulated that the s-triazine synthesis proceeded through the formation of biuret with spark discharges through the ice matrix containing brines of highly concentrated urea. This reaction is equivalent to the classic cyanuric acid synthesis from urea at high temperature[26], suggesting that s-triazines could be formed in dehydrating urea solutions through the formation of biuret.

It is unlikely that eutectic or concentrated urea solutions in a prebiotic setting were formed by pure urea. Cyanide in a dry/wet scenario in the presence of ammonia in oxidative environments could be an efficient prebiotic source of urea[14]; however, the irradiation of an ammonium cyanide solution would lead to a mixture of urea and guanidine, and it is possible to verify the formation of a viscous solution in the heating/dry-wet experiments (Fig. S2). Guanidine could represent a prebiotic precursor of 2,4,6-triaminopyrimidine (TAP), a non-canonical nucleobase that readily forms N-glycosides and C-glycosides with ribose and other aldoses[12]. Although TAP was not previously reported in prebiotic model reactions, the condensation of guanidine and malononitrile to yield TAP (and melamine, from self-condensation of guanidine) was observed to happen at high yield in dry conditions at T>100 ºC, as a mixture of TAP and melamine (Fig. S3). Moreover, in the more prebiotically relevant conditions of dry-wet cycles at 85 ºC, a mixture of urea and guanidine to which malononitrile has been added yielded TAP, melamine, and related amino-hydroxypyrimidines (Fig. S4); given that malononitrile could be a prebiotic precursor produced from cyanoacetylene[22], and malonic acid produced by irradiation of urea solutions[13,21], could malonic acid be a precursor of pyrimidines in the warm little pond prebiotic scenario? Considering that guanidine is a likely prebiotic product along with urea, a solution containing 2 mmol of urea and 1 mmol each of guanidine and malonic acid were subjected to dry-wet cycle under the same conditions previously detailed with urea or biuret alone (Fig S1). After 5 days of

wet/dry cycles at 65 ºC, a mixture containing pyrimidines was formed (Fig. 1) along with the formation of co-crystals (melting point 240-260 ºC), whose analysis by GC-MS shows are formed by barbituric acid and 2-amino-4,6-dihydroxypyrimidine, consistently with the melting point (Fig. 1B).

This reaction reached a maximum yield of 55% 2-amino-4,6-dihydroxypyrimidine, calculated from chromatographic peak areas and expressed as molar percentage of total malonic acid introduced in the experiment; as accompanying products, significant formation of 2,4,5-triaminopyrimidine, melamine, and a small amount of TAP were observed (Fig 1C). The reaction proceeds optimally in the range 65-85 ºC, losing efficiency at higher temperatures due to the increase of malonic acid decarboxylation to acetic acid and CO_2. In parallel, an experiment performed in the same conditions with urea alone in absence of guanidine, shows barbituric acid as main product, with an increased yield of 6-aminouracil respect to the experiment with guanidine (Fig. 1D); note the absence of cyanuric acid, which seems to be formed only when the carbamoylurea (biuret) is present in the experiment (Fig. S1)

incorporated by other processes. One significant aspect is the formation of the 2,4,5-triaminopyrimidine (Fig.1), identified through electron impact mass fragmentation signatures in GC-MS (Fig. S8) and consistent retention times with reference samples; this constitutes a possible prebiotic synthesis of this pyrimidine, which has also been proposed as possible building block of pre-RNA[27]

Urea and guanidine are essential in these reactions (Scheme 3), and the two possible urea alteration pathways in solution involve its decomposition into ammonia and carbon dioxide, and its isomerization to ammonium cyanate. The released NH_3 could lead to the formation of monoamidomalonic acid (observed in the experiments with malonic acid, Fig.1), and malonodiamide. The carbamoylation of monoamidomalonic acid by urea-derived isocyanate, followed by cyclization through intramolecular nucleophilic attack of the amino group, could explain the synthesis of the 4-amino-2,6-dihydroxypyrimidine. Finally, the condensation of malonodiamide (derived from malonic acid or malononitrile) with urea or guanidine, lead to the formation 2-hydroxy-4,6-diaminopyrimidine and TAP (scheme 3).

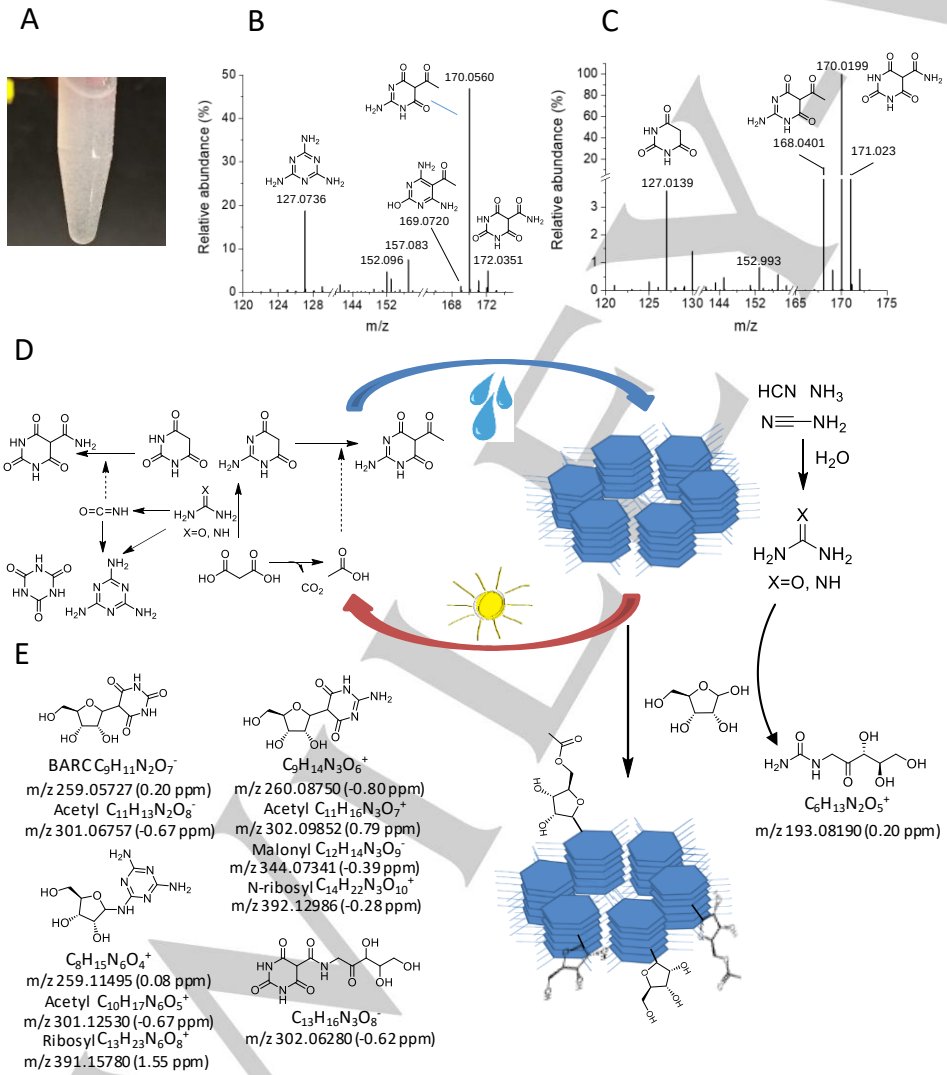


Figure 3. (A) Reaction tube showing formation of a pyrimidines precipitate after dilution with water of the product of wet-dry cycles at 85ºC of a urea/guanidine solution to which malonic acid was added; (B) Positive ion mode electrospray mass spectrum obtained after dissolution of precipitates in an ammoniacal water-methanol solution. (C) Mass spectrum (negative ion mode) of the precipitate dissolved in ammoniacal water-methanol, showing the exact masses of identified compounds. D: Dry-wet reaction model. Dehydration of the urea rich solution led to the condensation of urea and malonic acid, forming pyrimidines. Furthermore, decomposition of urea into isocyanic acid could be the key step in the formation of 5-carbamoylpyrimidines and the triazines. In the next phase of rehydration and dilution, previously formed pyrimidines could form supramolecular aggregates by base pairing (blue hexagons) that precipitate. An increase of concentration of urea, by evaporation or input of urea by hydrolysis of hydrogen cyanide or cyanamide, could dissolve the aggregates totally or partially. Hence, base pairing and dry-wet cycles could be a selection and concentration mechanism of nucleobases formed previously in urea solutions; (E) Experimental m/z, mass error and possible structures to relevant products found in the reaction between ribose and nucleobase aggregates.

Hence, the experiment suggests that condensation of malonic acid in the urea/guanidine mixture, subjected to environmental cycles at moderate temperature, could generate trisubstituted pyrimidines in plausible prebiotic conditions. This milieu and environment would be favorable for the capture of sugars

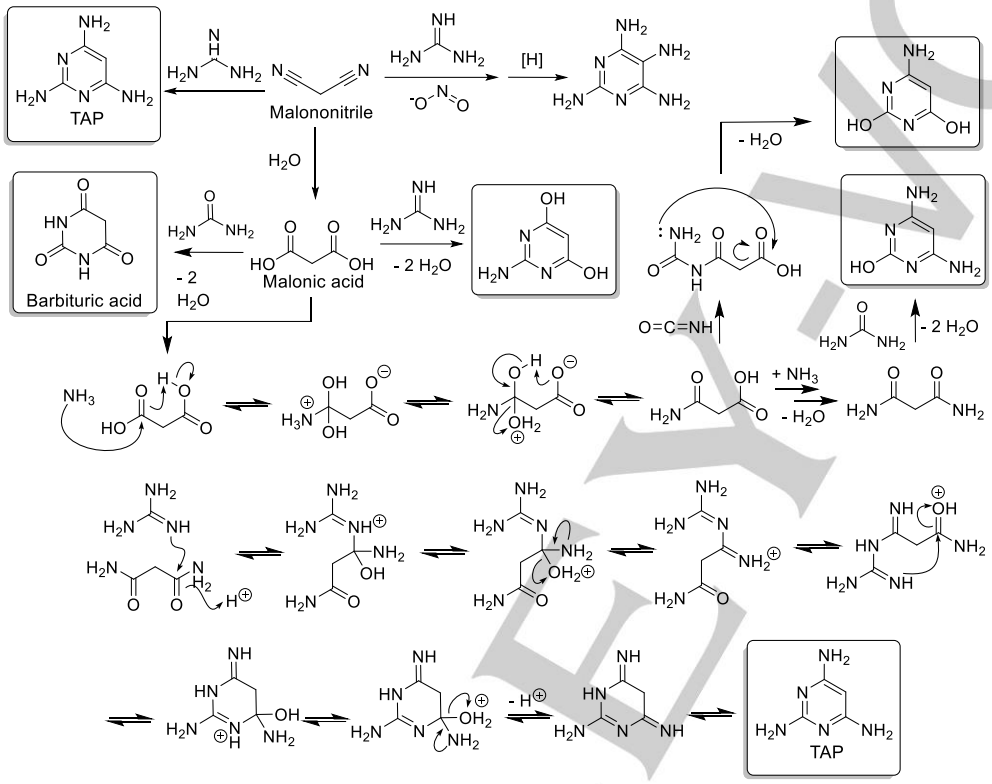
The weaker electrophilicity of an amide carbonyl compared with malonic acid is consistent with the observed yields, and with the preference for 2-amino-4,6-dihydroxypyrimidine in presence of guanidine (Fig.1). In support of this proposed reaction pathway,

a guanidine solution with malondiamide added showed TAP production by mass spectrometry after 3 days of dry/wet cycles at 85ºC (Fig. S5).

These results show that the synthesis of 2,4,6-trisubstituted pyrimidines is possible in mild, prebiotically plausible conditions, by the condensation of malonic acid with urea or guanidine in a simple evaporative setting; this dry-wet warm little pond model, leads to a scenario favorable for the prebiotic local concentration of relevant pyrimidines through precipitation/crystallization.

Dilution of the reaction mixture with water after 2 days of cycles results in the formation of a flocculent precipitate, which is observed to dissolve in a concentrated urea solution, re-precipitating by dilution. The separation of the precipitate and its analysis by Orbitrap mass spectrometry, after dissolution in a methanol-water-ammonia solution, revealed the formation of barbituric acid, melamine, and the acetyl and carbamoyl derivatives of these bases. The fragmentation of *m/z* 170.0560 in positive mode (Fig. S6a) suggests it corresponds to the acetylated pyrimidine at C5 (expected due to stabilization by its aromatic ring) rather than the acetyl ester. The temperature is an important factor in the process, as the formation of the precipitate and the acetyl and carbamoyl derivatives is favored at of 85 ºC, whereas at 65 ºC the crystallization of bases is preferred (Fig. 2B).

Overal, the resulting composition in the range 65-85ºC is similar, but increased temperature favors both the decarboxylation of malonic acid to acetic acid and the formation of isocyanic acid from urea. As a result, the experiments performed at 85ºC shows an increase in acetylated and carbamoylated pyrimidines, as well as in melamine. The formation of 2-amino-4,6-dihydroxy-5-acetyl pyrimidine is preferred compared to the formation of acetyl esters by resonance stabilization. It is interesting to note that the precipitation of supramolecular aggregates is favored when acetyl and carbamoyl derivatives are present; nevertheless, it could be more easily explained by the increase of formation of melamine, whose insoluble aggregate with barbituric acid derivatives precipitates even at very low concentrations. Hence, the urea solution in a wet-dry scenario at moderate temperatures provides a single environment for the synthesis and selection and concentration of nucleobases through the formation of supramolecular aggregates by base-pairing (case of barbituric acid/melamine, Fig. 3), coprecipitation with aggregates, by trapping in the flocculent precipitates, or formation of other low solubility structures (case of barbituric acid-TAP precipitate, for which it has not been demonstrated the formation of hexameric rosettes).



Scheme 1. The versatility of malononitrile. Formation of the barbituric acid family of pyrimidines by urea/guanidine and malonic acid condensation, driven by the decomposition/isomerization of urea in ammonia and isocyanic acid; the condensation of resulting mono and diamides of malonic acid with guanidine or urea lead the corresponding pyrimidines. One possible origin of malonic acid is the hydrolysis of malononitrile, which, in turn, could condense directly with urea/guanidine to yield the 4,6-diaminopyrimidines, or, in presence of atmospheric NO, form 4,5,6-triaminopyrimidines, as observed by Becker et al.[28]

The pyrimidines of the barbituric acid family could have played a key role in chemical evolution through the spontaneous formation of supramolecular aggregates and C-glycosides by reaction with aldoses, preferential at the C5 ring position, even in the presence of amino groups (in the instance of TAP). Considering an input of sugars from external sources, the presence of pyrimidine bases could harvest the reactive aldoses, resulting in the concentration of these nucleosides through the formation of insoluble supramolecular aggregates. The synthesis of sugars or nucleosides in the urea-rich scenario is unlikely, due to the reactivity of urea with aldehyde precursors (impairing formose reaction) or glyoxylic acid (inhibiting the glyoxylate scenario) and forming the corresponding urea derivatives. If aldose-reactive nucleobases were accumulated as aggregates as a form of *prebiotic organic mineral*, further input of ribose could lead to the formation of nucleosides and gradually concentrated it, preserved in the form of aggregates. To test this concept, we collected by centrifugation the precipitates formed by condensation of malonic acid in a viscous urea/guanidine mixture and heated the precipitates with ribose in solution at 70ºC for 6 hours. In a similar experiment, after wet/dry cycles of a urea/guanidine and malonic acid solution, ribose was added during the last wet/dry cycle, with no previous separation of precipitates. The products of both of these reactions were

analyzed by mass spectrometry, showing the exacts masses corresponding to BARC (confirmed by fragmentation and exact mass, after a control experiment showed in Fig. S7), 5-ribosyl-2-aminopyrimidine-4,6-diol, C-nucleoside of 2,4,6-triaminopyrimidine (TARC), and N-riboside of melamine (Fig. 3). It is interesting to note that the formation of monoacetyl-nucleosides is common, as confirmed by observation of the exact m/z of monoacetyl derivatives of all identified nucleosides (Table S1). Regarding the formation of malonic acid esters, we only identified the malonyl ester of 5-ribosyl-2-aminopyrimidine-4,6-diol. The formation of dicarboxylic acid esters of nucleosides could be an alternative pathway to the formation of nucleoside oligomers but, in this case, malonic acid is too prone to decarboxylation, leading to the observed acetyl esters. The formation of C-nucleoside of 2-aminopyrimidine-4,6-diol and TARC are demonstrated by the identification of the exact m/z of the corresponding di-ribosides. All characterized species formed are listed in Supplementary Table 1. The resulting qualitative composition in both experiments is similar. As expected, the addition of ribose to the urea-rich pond in one-pot without previous separation of precipitates, lead to ribosyl-urea (Fig.3E) and unidentified species. Hence, the separation and stabilization of non-canonical nucleobases in a small pond, which gradually concentrated and accumulated nucleobases and nucleosides by a simple selection mechanism, could have been a pathway to the collection of reactive sugars from different sources and the selection of nucleosides as building blocks for a potential pre-RNA molecule.

In summary, we showed that the condensation of malonic acid and urea or urea/guanidine in a prebiotic scenario, consisting in evaporating warm ponds at moderate temperatures, lead to a mixture of pyrimidines and triazines; furthermore, it leads to the spontaneous selection and concentration of nucleobases or nucleosides, if a suitable sugar is present. This process is mediated by the spontaneous glycosylation and formation of supramolecular assemblages by base pairing and base coprecipitates. A *little warm pond* enriched in urea could lead to a prebiotic mechanism of formation, concentration and selection of pre-RNA building blocks, powered by cycles of synthesis, precipitation, and redissolution, associated to environmental dry/wet cycles in the surface of early Earth.

Acknowledgements

This work was supported by NSF and the NASA Astrobiology Program under the NSF Center for Chemical Evolution (CHE-1504217). We thank Prof. Charles L. Liotta for his support facilitating the GC-MS analyses and fruitful discussions.

Keywords: Prebiotic Chemistry • Urea • Origins of Life • pyrimidines • nucleosides

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10.1002/cbic.202000510

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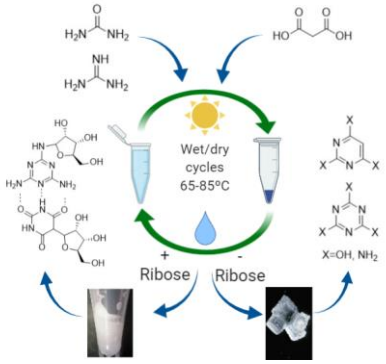

Concentrated pools of water enriched with urea would have been an efficient source for the formation of non-canonical pyrimidines. In the presence of ribose, or other aldose sugars, the bases derived from these urea-rich pools could react with these sugars through spontaneous glycosylation, ultimately concentrating these de novo nucleosides through aggregation. We demonstrate how a setting rich in urea and guanidine could lead to the synthesis and concentration of non-canonical nucleosides on a prebiotic Earth.

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