A possible prebiotic function of cytosine as amino acid synthesizer

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When cytosine goes alanine...

ABSTRACT The biochemical role of purine and pyrimidine bases may have been very different at the beginning of life. Their possible direct involvement in amino acid synthesis was examined via development of an in vitro reaction system, which simulated the chemical constituency of early life microenvironments. Reductive amination of pyruvate to alanine and possible alanine products was carried out under anhydrous conditions. A similar role for the adenine/hypoxanthine couple could not be verified. The possible evolutionary precedence of such an imine-involving mechanism for direct amino acid synthesis to the appearance of nucleic acids as ‘genetic information conveyors’ is proposed.

INTRODUCTION It is generally believed that life first appeared in a very hot, acidic, reducing, anaerobic, aqueous environment rich in hydrogen gas and sulfur and iron compounds of very negative redox potential1-3. Free nucleic acid bases can be synthesized from aqueous ammonium cyanate or from a mixture of methane, ammonia and water4, but also from formamide5-6. The free bases are of low solubility, not only to water but also to organic solvents7, while ribonucleosides are water soluble. Under the harsh prebiotic conditions of early life, free bases probably existed initially rather than ribonucleosides, as indicated by the ease of nucleic acid depurination8 and the cleavage of N-glycosidic bonds9 under hot acidic conditions, and ribonucleosides and nucleic acids evolved later. It is difficult to envisage how nucleic acids could ‘code’ for protein synthesis in their very early evolutionary stages when they are expected to have been small, perhaps only triads of bases, and devoid of regulatory promoter-like and ribosome-binding sites.

It is believed that RNA is the earliest form of nucleic acid and DNA appeared later, as a more stable depository format of the genetic material10-13. Therefore, to relate the base structure to any probable catalytic role in amino acid synthesis, we could consider the four RNA bases. Thymine is 5'-methylated uracil and cytosine is uracil aminated at carbon 4. Uracil and cytosine may be considered two alternative states of the same base, with cytosine donating its 4-amino group for reductive amination of an α-keto acid to the corresponding amino acid, and being converted into uracil in the process. A similar role can be envisaged for hypoxanthine and its 6-aminated state, adenine.

This article attempts to experimentally prove such a directly biosynthetic role of pyrimidines in specific prebiotic microenvironments, at the very early stage of small, perhaps even trinucleotide-size RNAs or even at a free base stage. The functional role of the exocyclic amino group of cytosine in aminating pyruvate to alanine was examined. Pyruvate may be an early prebiotic organic molecule, formed in hydrothermal vents from alkyl thiols and carbon monoxide, under the catalytic action of iron-sulfur centers14. According to a prebiotic evolution hypothesis15-17, amino acids were formed first and polypeptides followed by random polymerization. The ‘RNA world’ theory18 proposes that RNA was formed first by random polymerization of ribonucleotides. Selection for peptidyl transferase activity, arising from the need for better catalysis in ribonucleoprotein complexes, resulted in the first ribosomes and polypeptide synthesis. This work supports the view that at some evolutionary point amino acids may have started to be synthesized in a way dependent on the bases, which could be the origin of the contemporary interdependence of polypeptide-nucleic acid synthesis. This eliminates a significant degree of randomness, since it creates spatial proximity of amino acids to bases, facilitating a subsequent polymerization into polypeptides and RNA, respectively.

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Illustration by Eloïse Kremer
The alanine-cytosine conjugate is subsequently sulfonated by sodium acid sulfite (added as sodium bisulfite). A reaction scheme for the use of cytosine in the amination of pyruvate to alanine was designed, as described in the Methods, and is shown in Figure 1.

A purple spot of retention factor (Rf) the same as that of alanine was produced using the ninhydrin reaction on paper chromatograms of the cytosine-pyruvate reaction (Figure 2). Purple color is produced with amino acids that have a primary amine group, whereas proline and imino acids give yellow products. The method is very sensitive, being able to detect picogram quantities of amino acids. The purple spot was produced with greater efficiency when a combination of Zn and HCl were used as the in situ hydrogen-generating system. A minimum of 1 h of hydrogenation and overnight incubation at 4°C, after sulfonation, were required for the amino acid spot to be detected. Only the cytosine-pyruvate reaction produced the purple spot.

The proton nuclear resonance (H-NMR) spectra (Figure 3) of the supernatants of the two reactions show a broad peak at 5.5 ppm, characteristic of the alanine amino group (NH$_2$)$_3^+$ present only in the cytosine-pyruvate reaction. It cannot be attributed to the cytosine exocyclic 4-amino group, which resonates at >7 ppm.

The two-dimensional homonuclear correlated nuclear magnetic resonance (H-H-COSY NMR) spectra (Figure 4) both show coupling (solid line rectangle) of a methyl group at 1.22 ppm to a 3.6 ppm peak. These values can be attributed to the lactate salt methyl-

amine moiety (CH$_2$-CH$_2$-) resonance at 1.38 ppm, and a 3.4 ppm peak (dotted line square) is evident in the cytosine-pyruvate reaction and could account for the strong positive ion peak of m/z = 186 (cytosine-alanine-H$^+$) with retention time 4.72 min. Small amounts of an m/z = 112 peak at 4.6 min, attributed to the alanine-Na$^+$ positive ion, were also detected only in the cytosine-pyruvate reaction. Similarly, an m/z = 132 ion at 5.4 min, which could represent N-acetyl-alanine-H$^+$ ions formed by reaction of alanine with pyruvate degradation products, showed only in the cytosine-pyruvate reaction. A prominent peak of m/z = 325, at 5.75 min of the cytosine-pyruvate chromatogram, fits the mass of a tetra-alanine-Na$^+$ positive ion.

Liquid chromatography-mass spectrometry (LC-MS) analysis (Figure 5) detected the positive ion of alanine (alanine + H$^+$) at m/z = 90, in the cytosine-pyruvate reaction (‘C’), but not in the uracil-pyruvate reaction (‘U’), at retention time 4.18 min, the same as that of standard alanine. Only small peaks, of noise level intensity, are detected at the same retention time of the uracil-pyruvate reaction chromatogram.

Other peaks, unique to the cytosine-pyruvate reaction, appear in the spectra as shown in Figure 6. Both reactions show large amounts of m/z = 91 peaks at retention time 3.5-4.7 min, which can be attributed to lactate-H$^+$ positive ion. The alanine-cytosine conjugate is expected to occur only in the cytosine-pyruvate reaction and could account for the strong positive ion peak of m/z = 186 (cytosine-alanine-H$^+$) with retention time 4.72 min. Small amounts of an m/z = 112 peak at 4.6 min, attributed to the alanine-Na$^+$ positive ion, were also detected only in the cytosine-pyruvate reaction. Similarly, an m/z = 132 ion at 5.4 min, which could represent N-acetyl-alanine-H$^+$ ions formed by reaction of alanine with pyruvate degradation products, showed only in the cytosine-pyruvate reaction. A prominent peak of m/z = 325, at 5.75 min of the cytosine-pyruvate chromatogram, fits the mass of a tetra-alanine-Na$^+$ positive ion.

As further supporting evidence for the proposed putative identity of these additional products, unique to the cytosine-pyruvate reaction, the UV absorption
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DISCUSSION

Synthesis of amino acids by reductive amination of \( \alpha \)-keto acids by ammonia in an aqueous environment is possible\(^{25,26} \), but the amino acids are N-acylated by a second molecule of the \( \alpha \)-keto acid and hydrolysis at pH 3-4 is required to liberate them. This work shows that amino acid synthesis can occur in a hydrophobic, reducing microenvironment. The imine synthesis step requires an anhydrous environment, achieved by carrying out the reaction in xylene. An anhydrous microenvironment around an \( \alpha \)-keto acid could exist if several purine or pyrimidine molecules, forced by the repulsive forces of the surrounding water, could associate to form a hydrophobic cavity containing the \( \alpha \)-keto acid, as happens in the interior of the nucleic acid double helices. As has been proposed\(^{27} \), the formation of ribonucleosides and then oligonucleotides would facilitate the bases staying together and the formation of such a hydrophobic cavity, establishing the three-base codon as the minimum required size for the enclosure of \( \alpha \)-keto acids and their amination to amino acids.

Amination of \( \alpha \)-keto acids using free ammonium ions and reductive power from sulfur compounds, such as FeS and H\( _2 \)S, without the need for the existence of any nucleic acids or even bases, has been experimentally proved by Hafenbradl et al.\(^{28} \). Similarly, Huber and Wächtershäuser\(^{29} \) have reductively aminated a variety of \( \alpha \)-keto acids into amino acids, using ammonium ions and FeS. In their reactions, aqueous media were used and the pH was 8.8-12.7, which is close to the pKa of the ammonium ion source. Their proposed primordial amino acid synthesis scheme fits well with an alkaline aqueous early life environment. In contrast, the model we propose in this paper requires hot, mildly acidic, sulfurous, hydrophobic microenvironments, such as those in the interior of a nucleic acid double helix or in the interior of cytosine-hydrophobic compounds.
aggregates, positioned close to undersea hydrothermal vents. The presence of $H_2$ and ammonia, as well as the low pH and high temperature in hydrothermal vents, are well documented$^{30,32}$. The reaction conditions used in this work are similar to the conditions encountered in hydrothermal vents. The only disputable point could perhaps be the use of bisulfite ion $HSO_3^-$ used in sulfonation. In a water, the bisulfite ion is in chemical equilibrium with $SO_2$. Although (bi)sulfite and $SO_2$ are present in magma and volcano emissions$^{33,34}$, they have not been reported for submarine hydrothermal vents$^{31}$. This is expected because $SO_2$ is rapidly converted into bisulfite ions upon contact with water. Bisulfite itself readily reacts with dissolved oxygen to give sulfate ions. Its existence in hydrothermal microenvironments under oxygen-free prebiotic conditions is a reasonable expectation.

A previous hypothesis on the involvement of the exocyclic groups of cytosine, adenine and guanine in amino acid synthesis is probably incorrect. The reaction of amino acid ketone esters under the experimental conditions we used. In adenine, the positive charge of the initial carbocation, formed during the sulfonation step, is attracted to the amino group of a second amino acid, forming a peptide bond. However, there are no alcohols in our reactions for such ester formation, except the lactate hydroxyl group, after the reduction step (Figure 1) and the possibility must be examined that lactate could be involved in peptide formation.

Alternatively, under the dehydrating conditions of the reaction, before the sulfonation step, anhydrides could form between the carboxyl groups of two alanines still conjugated to cytosines. Such anhydrides can be attacked under anhydrous conditions by the secondary amino group of a third alanine, still bound to cytosine. The resulting dipeptide will have both its amino groups still attached to cytosines, from which it can detach itself after sulfonation. Also, two such dipeptide-cytosine conjugates can further react similarly to produce tetra-peptides. No longer peptides have been detected. This route of amino acid polymerization into peptides has previously been found to be successful$^3$ and is shown in Figure 8.

Adenine was shown to be unable to aminoate keto acids under the experimental conditions we used. In adenine, the positive charge of the initial carbocation, formed during the sulfonation reaction, spreads to the neighboring imidazole ring, making attack by the nucleophile $HSO_3^-$ more difficult. The same, of course, holds for the case of guanine. The development of special conditions, resembling those of ancestral life, will have to be designed for proving a similar
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function of purines in amino acid synthesis or a different primeval role may have to be proposed for them. Uracil and hypoxanthine (the precursors of adenine and guanine) possess no exocyclic amino group to donate to pyruvate and can be considered deaminated forms of cytosine and adenine, respectively. Accordingly, uracil could be seen as an initial acceptor of amino and other nitrogen-containing groups, being converted into cytosine in the process. A possibility presently examined by our team is that purines evolved later than pyrimidines and were used as the initial harvesters of inorganic nitrogen species, other than ammonia, subsequently passing these groups to uracil.

CONCLUSION

The amino acid alanine can be synthesized by reductive amination of the corresponding $\alpha$-keto acid pyruvate by cytosine, in a hydrophobic, reducing microenvironment, without the need for extreme pH. This is proposed as a possible primeval route to amino acid synthesis and as a potential first function of nucleic acids or free pyrimidines, before their role as ‘genetic information’ molecules was evolved.

METHODS

Formation of the Schiff base

A combination and adaptation of the methods of Kundu et al. and Khrushcheva et al. were used for the formation of imines between pyruvic acid and pyrimidines.
Two to 100 mg of pyrimidine base (cytosine or uracil) were separately put into a 1.7 mL microfuge tube, together with an equal weight of sodium pyruvate and anhydrous sodium sulfate. The mixture was suspended in 500 μL xylene and heated in a microwave oven (Deawoo model KOR-6135) three times, for 3 min each time, at maximum power. The reactions were then left at 90°C for 24 h. Imine synthesis requires anhydrous conditions and is known to proceed also in solid phase reactions. Xylene was used to facilitate the heat transfer processes in the reaction mixture and the reaction ingredients are insoluble to it. Sodium sulfate was included to help remove traces of water present in the reactants or generated during the Schiff base formation. At the end of the reaction period, the mixtures had the appearance of a white paste, with most of the xylene and water having evaporated.

**Reduction of the imine N=C bonds into amine and liberation of the amino acid**

After completion of the imine formation reactions of the previous section, 10 mg solid zinc powder and 250 μL of xylene were added to each of them. Then, 10 μL 2M HCl were added under the xylene layer and mixed gently with the zinc-pyruvate-base solid mixture. The reaction was allowed to proceed at 60°C for 3 h, with the lids of the microfuge tubes open. Evolution of hydrogen gas bubbles trapped under the xylene layer among the solid constituents was visible after 15 min. In separate reactions, reduction with 10 μL 1 M LiAlH₄ in 2 M HCl was also attempted. After 3 h, all of the water and the xylene had evaporated, leaving a dry, white solid, which was light yellowish in the case of the cytosine reaction.

To split the base-amino acid conjugate into free base and amino acid, the bisulfite reaction was adapted. To the solids of each pyrimidine reaction, 300 μL freshly made 2 M NaHSO₃ (as sodium bisulfite) were added, and the mixture was briefly vortexed and allowed to stand at 4°C overnight. The sulfonate of the secondary amine (base-alanine complex) is insoluble to it. Sodium sulfate was included to help remove traces of water present in the reactants or generated during the Schiff base formation. After a 10-minute heating at 90°C to help dissolve the products, any remaining insoluble matter was pelleted at 10,000 x g for 15 min. The supernatant, containing any produced alanine and other solubles, was transferred to a clean microfuge tube.

**Detection and identification of soluble products**

Five μL of each reaction were analyzed by ascending paper chromatography with butanol/acetic acid/water 12/3/5 (v/v/v) (alanine Rf = 0.21) as the developing phase, for a distance of 20 cm. The chromatogram was then briefly dipped into a solution of 0.1 w/v ninhydrin in acetone and heated to 100°C for 5-10 min. Purple (mauve) spots, characteristic of amino acid reaction with ninhydrin, were developed at an Rf the same as that for the alanine standard. The rest of the water soluble reaction products were allowed to dry under reduced pressure, redissolved in 500 μL of water and 20 μL of the mixture were subjected to LC-MS using a LCMS-2010EV Shimadzu instrument, equipped with a 4.6 x 150 mm Pathfinder silica 100, 3.5 UM reverse phase HPLC column. The liquid phase consisted of 50% v/v methanol, 50% v/v water, 0.05% v/v formic acid, at a flow rate of 0.4 mL min⁻¹.

Detection of the eluted compounds was via a SPD-M20A diode array detector, scanning at all wavelengths from 190 to 800 nm, and by a MS detector using an electrospray interface (ESI) in positive ionization mode at full scan acquisition between m/z 50-500. The detector voltage was set at 1.4 kV and the nebulizing gas (N₂) flow rate was 1.5 L min⁻¹. These conditions preserve most of N+1 molecular positive ion (with proton addition) and the N+23 positive ion (with sodium ion addition). For interpretation of the mass spectrum, unique to the cytosine-pyruvate reaction (Figure 6)
Figure 8 | Percentages of the original pyruvate transformed into other products. Putative pathways for tetra-alanine formation via an ester to lactate and via an anhydride are also shown.

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CONFLICTS OF INTEREST Authors declare no conflicts of interest.

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REFERENCES