Prebiotic Chemistry Hot Paper

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A Plausible Simultaneous Synthesis of Amino Acids and Simple Peptides on the Primordial Earth**

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Dedicated to Stanley L. Miller and Joan Oró

Abstract: *Following his seminal work in 1953, Stanley Miller conducted an experiment in 1958 to study the polymerization of amino acids under simulated early Earth conditions. In the experiment, Miller sparked a gas mixture of CH_4, NH_3, and H_2O, while intermittently adding the plausible prebiotic condensing reagent cyanamide. For unknown reasons, an analysis of the samples was not reported. We analyzed the archived samples for amino acids, dipeptides, and diketopiperazines by liquid chromatography, ion mobility spectrometry, and mass spectrometry. A dozen amino acids, 10 glycine-containing dipeptides, and 3 glycine-containing diketopiperazines were detected. Miller's experiment was repeated and similar polymerization products were observed. Aqueous heating experiments indicate that Strecker synthesis intermediates play a key role in facilitating polymerization. These results highlight the potential importance of condensing reagents in generating diversity within the prebiotic chemical inventory.*

Stanley Miller published the synthesis of amino acids by sparking a gas mixture of methane, ammonia, water, and hydrogen,[1] which were considered in the early 1950s to be representative of the early Earth's atmosphere.[2] Today, however, a weakly reducing or neutral primitive terrestrial atmosphere comprised of major constituents such as CO_2, N_2,[3] CO, and H_2O, with minor components, including reduced gases such as H_2, H_2S, and CH_4,[4] is favored to a strongly reducing gas mixture. Although reducing atmospheric conditions may have been unlikely on a global scale on the early Earth, they might have been present on smaller scales[5] that could have been important locales capable of fostering a suite of very powerful prebiotic chemical reactions to produce large quantities of molecules important for life.[5a,b] Laboratory studies have shown that, even under neutral conditions, amino acid synthesis is efficient.[4]

A combination of Miller's pioneering 1953 experiment[1] and the subsequent findings of extraterrestrial organic compounds in meteorites[6] indicates that the synthesis of prebiotic organic compounds thought to be necessary for the origin of life is a robust process, both on the primitive Earth and on other planetary bodies.[7] However, the transition from simple molecules, such as amino acids, to more complex ones, such as peptides, has proven challenging under plausible primordial conditions. Although the syntheses of peptides by hydrothermal vents and comet impact have been reported, questions remain about their plausibility under prebiotic geochemical conditions.[4] In addition, concentrated salts, clays, and Cu^{2+} ions have been suggested as being important amino acid condensation reagents,[8] although these have not been demonstrated to be effective polymerization agents under the natural geochemical environments that may have existed on the early Earth. For example, Cu^{2+} ions in the primitive oceans would have been in the form of Cu^{+} and its concentration would have been very low because of the presence of HS^{-}.[9] Additionally, other reduced metal ions,

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such as Fe^{2+}, may have been present[10] and could have played a role in shaping prebiotic environments and the chemical reactions that may have occurred therein. Other potential prebiotic polymerization agents such as carbonyl sulfide (COS) have been proposed,[11] although the overall efficiency with respect to the variety of amino acids that can undergo polymerization with this reagent has not been explored.

In carbonaceous meteorites, the timescale for the production of amino acids is typically estimated to be between 10^3 and 10^6 years,[12] but could be as short as 1–10 years.[13] The prebiotic chemistry that took place on the meteorite parent bodies during the aqueous alteration phase is considered to have produced mainly simple monomeric compounds[7a] and complex, poorly characterized, polymers.[14] Only very low, trace, quantities of glycine dipeptide and its diketopiperazine (DKP) have been detected in a few cases.[15]

Recently, archived stored portions of the solutions from experiments Miller carried out in 1958 were found.[5b] Included in this set were labeled vials from an experiment in which reduced gases (methane and ammonia) were subjected to a spark discharge for about 7 days, and over the course of the experiment, cyanamide was intermittently added to the aqueous phase. These archived cyanamide samples were part of a large collection of samples that Miller saved from a number of his experiments in the 1950s.[7a] For unknown reasons, Miller never performed a chemical analysis of the products of the cyanamide experiment and others conducted in 1958.[5b]

Although it has not yet been demonstrated that cyanamide can be formed within electric discharge experiments, the production of cyanamide in plausible prebiotic conditions comprised of CH_4, NH_3, H_2O, and UV light was reported nearly 50 years ago, and was proposed to be a possible prebiotic condensing reagent.[16] Preliminary experiments supported this scenario,[17] although the reaction seemed to be most favorable at acidic pH values lower than the pK_1 value of the amino acid.[18] This finding suggested that the reactive amino acid species is H_3N^+-C(RR′)-COO^- (where R and R′ represent the amino acid side chains). It has also been proposed that cyanamide can activate *N*-acyl-α-amino acids to form a 5(4*H*)-oxazolone intermediate that can help facilitate the coupling of sterically hindered α-amino acids.[19] In addition, cyanamide has been suggested to be influential in other important prebiotic reactions, such as the synthesis of activated pyrimidine ribonucleotides[20] and 2′-deoxynucleotides.[21]

Miller never carried out a detailed analysis of his 1958 cyanamide experiment, but he did measure the absorption at 280 nm when he collected various fractions during chromatographic separation of the discharge solution from the cyanamide experiment and found absorption in several samples where peptides were expected to elute.[22] We have now analyzed the 1958 cyanamide spark discharge residues to ascertain if both amino acids and simple peptides had actually been synthesized simultaneously in this prebiotic simulation experiment. Amino acids were analyzed using high-performance liquid chromatography with fluorescence detection and triple quadrupole mass spectrometry. Dipeptides and DKPs were identified and quantified using ultraperformance liquid chromatography coupled to quadrupole-traveling wave ion mobility spectrometry/time of flight mass spectrometry (for further details on the analytical tools used in this study, please see the Supporting Information).

The analysis of Miller's archived cyanamide experiment samples resulted in the detection of 12 amino acids, 10 glycine-containing dipeptides, and 3 glycine-containing DKPs (Table 1). The amino acids produced by the cyanamide experiment were synthesized in relatively high yields, and present in similar relative abundances compared to those detected in Miller's classic and volcanic,[5a] as well as hydrogen sulfide containing[5b] spark-discharge experiments (Figure 1). Major amino acids with stereogenic centers (e.g. aspartic and glutamic acids, serine, alanine, and isovaline) were racemic (D/L ≈ 1:1) within error limits (10%), thus

Table 1: Amino acids, dipeptides, and DKPs that were both identified and quantified in this study.

Amino acids[a]	Dipeptides[b]	DKPs
glycine	Gly-Gly	cyclo(Gly-Gly)
alanine	Gly-Ala	cyclo(Gly-Pro)
β-alanine	Gly-Thr	cyclo(Leu-Gly)
serine	Gly-Pro	
α-aminobutyric acid	Pro-Gly	
β-aminobutyric acid	Gly-Val	
γ-aminobutyric acid	Val-Gly	
aspartic acid	Gly-Glu	
glutamic acid	Glu-Gly	
valine	Leu-Gly	
isovaline		
isoleucine		

[a] Additional amino acids were tentatively identified, but were not quantified and, therefore, not included here, but are listed in the Supporting Information. [b] Additional dipeptides, as well as higher order peptides, such as the tripeptides Pro-Pro-Gly and Asp-Asp-Gly, were tentatively identified within the archived samples and are also shown in the Supporting Information. These initial identifications indicate that the formation of tri- and higher peptides in prebiotic simulation experiments warrants further investigation.

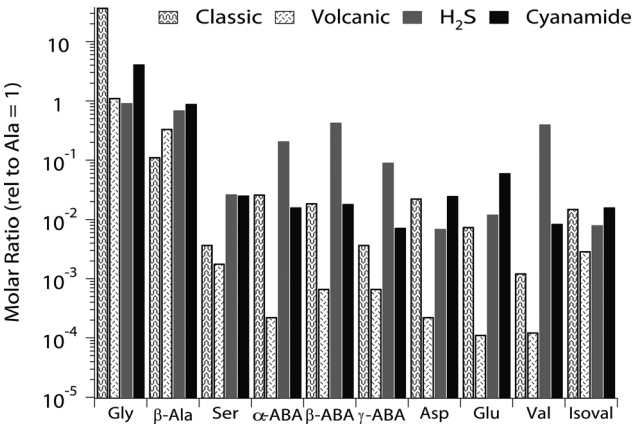


Figure 1. Molar ratios (relative to D + L-Ala = 1) of major amino acids in unhydrolyzed samples from the classic, volcanic, H_2S, and cyanamide spark-discharge experiments.

indicating their abundances were minimally influenced by contamination with terrestrial L-amino acids during sample storage and processing.

Glycine-containing dipeptides and DKPs were targeted for analysis. Glycine is the simplest amino acid and is one of the most abundant amino acids formed in spark-discharge experiments. Therefore, many peptides present in the samples reported here should contain glycine. Multiple analysis workflows (see the Supporting Information) were used to confirm the identity and quantity of the dipeptides and DKPs (Figure 2). The ratio of amino acids to dipeptides in the

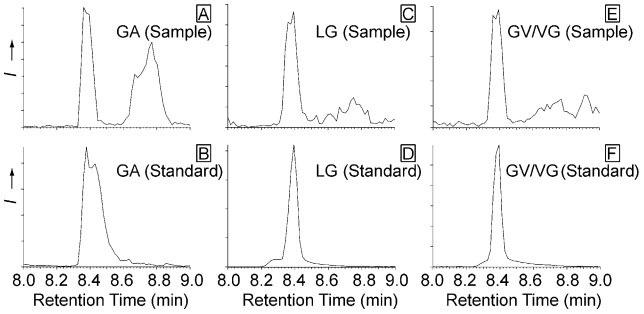


Figure 2. An example of how glycine-containing dipeptides were initially detected. Accurate-mass ultraperformance liquid chromatography (UPLC)/mass spectrometry analysis screened for specific dipeptide $[M+H]^+$ ions to provide a preliminary detection of the dipeptides of interest based on retention time and high-resolution exact mass, compared to standards. After this step, a more rigorous analytical approach was taken for unambiguous identification and quantification of the targeted dipeptides (see the Supporting Information). Extracted UPLC mass chromatograms, corresponding to the 8–9 min retention time window are displayed for specific dipeptides detected in Miller's sample fraction 57–67, and for standard traces. Chromatograms (A) and (B) were extracted by screening the total ion chromatograms for m/z 147.0770, C) and D) correspond to m/z 189.1239, and E) and F) correspond to m/z 175.1083. GA = glycylalanine, LG = leucylglycine, and GV/VG = glycylvaline/valylglycine.

cyanamide samples was calculated to be approximately 1000:1–1000:10, which agrees well with experimental data that indicates that the amino acid to dipeptide ratio is approximately 1000:1 under equilibrium conditions.[23] Furthermore, experimental data suggest that, at equilibrium, the dipeptide to DKP ratio should be on the order of 1:10,[24] and this ratio was determined to be 1:10–1:20.5 in the samples studied here. The cyclic nature of the DKP is responsible for its higher thermodynamic stability, and thus, greater abundance than the linear dipeptide at equilibrium.[24]

The presence of dipeptides in the archived samples was further confirmed by performing an acid hydrolysis on a portion of each sample,[25] analyzing the hydrolyzed fractions, and verifying that the peptide bonds had been cleaved to yield their amino acid residues. Additionally, identical dipeptide and DKP analyses, as reported for Miller's cyanamide samples, were carried out simultaneously on electric-discharge samples from Miller's 1958 hydrogen sulfide experiment,[5b] which did not incorporate a condensing reagent. Peptides were undetectable in the H_2S samples, thus providing added evidence to suggest that the presence of a condensing reagent facilitates the polymerization of amino acids.

In addition to investigating the archived cyanamide samples with modern analytical techniques, Miller's cyanamide experiment was repeated to generate fresh samples for further study. The analysis of the aqueous solution from the repeated experiment was compared to that of the original samples. The repeated experiment resulted in polymerization products, including dipeptides similar to those detected in the original 1958 cyanamide samples. These findings help corroborate the results obtained from the archived samples in suggesting that cyanamide can induce peptide formation under such a mimicked primitive Earth environment (the experimental and analytical details of this work are provided in the Supporting Information).

The formation of dipeptides in a mildly basic medium (pH 8–10) created by ammonia in the spark-discharge experiment contrasts with previous reports that indicate that acidic conditions are necessary to promote cyanamide-mediated polymerization of amino acids. As noted previously, in acid solutions with pH values less than the pK_1 value of the amino acid,[18] the reacting amino acid species would be H_3N^+-C(RR′)-COO^-. As the pK_a value of the COOH group is 2–2.5, the concentration of this reactive species decreases as the pH increases above pH ≈ 3, and the abundance of the protonated carboxylic acid is thus expected to be negligible at the pH value of the spark-discharge experiments. This suggests that perhaps one or more components intrinsic to the spark-discharge experiment may be responsible for facilitating the observed amino acid polymerization. Possible candidates include the amino acid amides and nitriles, both of which are intermediates in the Strecker reaction involved in amino acid synthesis.[7a,26]

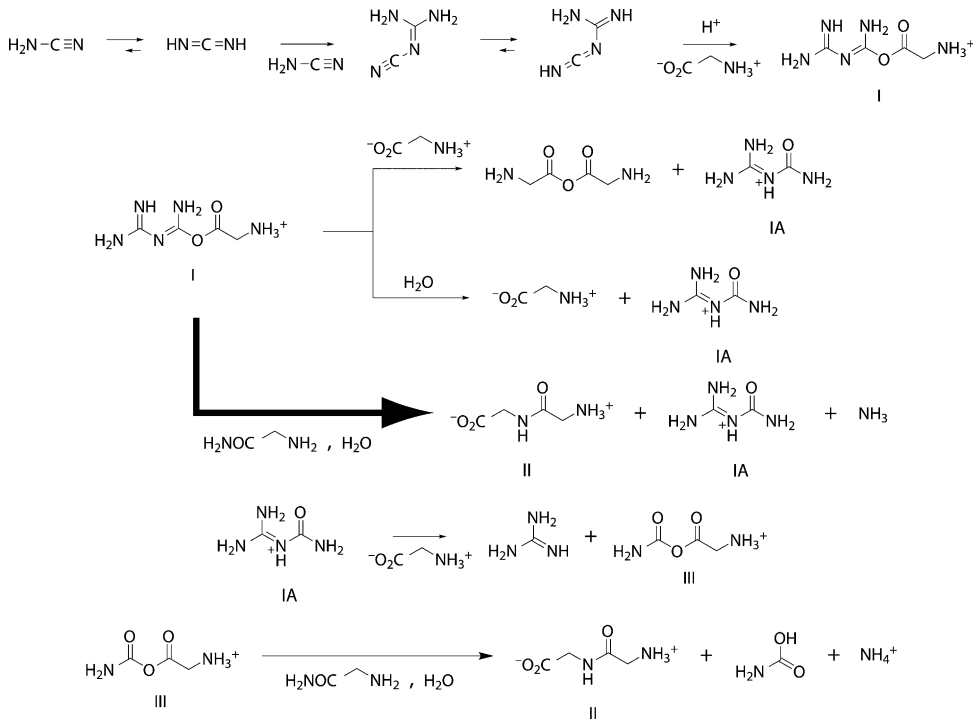
Heating experiments on aqueous solutions were carried out to evaluate how dipeptide synthesis could proceed under mildly basic conditions. Solutions containing only amino acids in the presence of cyanamide or its dimer, dicyandiamide (2-cyanoguanidine), were prepared at pH 1–2, pH 6–7, pH 9–10, and pH 12–13 and heated at 50 °C. Although dicyandiamide was not directly introduced into the discharge apparatus, its potential as a condensing reagent was evaluated because cyanamide is known to dimerize readily in basic solutions[27] and because dicyandiamide is also a proposed prebiotic condensing reagent.[28] Analyses of the heated solutions at various pH values confirmed that dipeptide synthesis only took place at acidic pH values. Next, other individual components, 1) ammonia, in the form of NH_4Cl, 2) amino acid amide, and 3) amino acid nitrile, were added separately to the solutions to better understand the synthetic route to dipeptides in the cyanamide spark-discharge experiment and to evaluate the possible roles of these species in facilitating polymerization. These solutions were analyzed for dipeptides and DKPs after being subjected to heating at 50 °C for times of up to 3 weeks. Solutions that were not heated were frozen at 0 °C for use as a ($t = 0$) control.

The presence of ammonia resulted in negligible quantities of polymerization, so its role can be eliminated. However, it was observed that at a mildly basic pH value, cyanamide and dicyandiamide reacted readily in the presence of the amino

acid amide, and a factor of 2–4 times less in the presence of the amino acid nitrile, to generate dipeptides (see Figure S3 in the Supporting Information). These results indicate that the presence of the amino acid amide, or amino acid nitrile, is involved in the cyanamide-mediated amino acid polymerization reaction. It should be noted that under these conditions it was observed that dicyandiamide facilitated the formation of twice the concentration of dipeptides than did cyanamide, therefore dicyandiamide performed as the superior condensing reagent. This suggests that in the electric-discharge experiment, the dimerization of cyanamide, which is fastest at pH 9.6,[27] close to the pH value of the repeated cyanamide experiment, may have produced dicyandiamide within the discharge solution, where it then likely played a greater role in initiating amino acid polymerization, than cyanamide itself.

Scheme 1, which is based in part on other studies,[29] shows a possible mechanism for the cyanamide-mediated synthesis of linear peptides at pH 9–10. Here, the carbodiimide form of cyanamide dimerizes to dicyandiamide, under mildly basic conditions, which can then be attacked by the nucleophilic carboxylate group of the amino acid to form the activated amino acid (I). At pH > 8, the amino group of glycinamide ($pK_a \approx 8$)[18] can attack the activated amino acid, the product of which can subsequently be hydrolyzed to ultimately give the linear dipeptide (II). Note that the pK_a value of the amino group of glycinamide is lower than that of the amino group of glycine, which is approximately 9.8.[18] As a consequence, the NH_2 group of glycinamide will be less protonated under such a regime, while the NH_2 group of glycine will be more protonated. Thus, glycinamide is a better nucleophile than free glycine in the pH regime of the spark-discharge experiment. However, it is worth noting that the unprotonated amino acid would also be a reactive species at pH values greater than the pK_a value of the amino group in glycine.

Hydrolysis of the amino acid amide to yield the amino acid is a potentially inhibitive pathway to dipeptide formation that should be considered. At pH = 9.75 and 55 °C, the half-life of glycinamide is about 3 days, while at pH = 7.95 and 75 °C, the half-life of glycinamide is about 7 days.[30] Extrapolating from these data, and considering that the cyanamide experiment was also mildly basic and that the reaction flask was no longer heated after the introduction of cyanamide (see the Supporting Information), glycinamide is expected to have had a sufficiently long lifetime to help facilitate the observed polymerization chemistry. Likewise, it is probable that the same is true in the case of the heating experiments that were performed that mimicked the spark-discharge solutions.



Scheme 1. Scheme showing the dicyandiamide-mediated reactions involved in the polymerization of amino acids. The main dipeptide formation pathway is highlighted by the bold arrow, whereby the attack of the amino acid amide on the reactive intermediate (I) first yields the peptide amide, which is then hydrolyzed[30] to give the linear dipeptide (II).

Also shown in Scheme 1 are several possible routes by which a second activated monomer (IA) can be formed as a by-product, which itself may undergo similar reactions as dicyandiamide to form the linear dipeptide. These possible additional dipeptide formation pathways may help explain why dicyandiamide induces more amino acid polymerization than does cyanamide.

The findings detailed here demonstrate the simultaneous synthesis of both simple and complex molecules under plausible prebiotic conditions. Miller's cyanamide experiment marks the first effort to study a prebiotic condensing reagent for its implications to the origin of life. Additionally, the results obtained here highlight the potential importance of condensing reagents in providing a mechanism to explain how simple organic compounds such as amino acids may have polymerized to form more complex molecules, such as dipeptides. The synthesis of dipeptides and DKPs by the cyanamide polymerization reaction may have additional implications, as some dipeptides and DKPs have been found to have catalytic properties that may have been important on the primordial Earth.[31]

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