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 CH₄, NH₃, and H₂O, 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, [3] 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 CO₂, N₂, [3] CO, and H₂O, with minor components, including reduced gases such as H₂, H₂S, and CH₄, [3] 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 [3] 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. [3] 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 Cu²⁺ 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, Cu²⁺ 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⁻ [4a] Additionally, other reduced metal ions,
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

<table>
<thead>
<tr>
<th>Amino acids[a]</th>
<th>Dipeptides[b]</th>
<th>DKPs</th>
</tr>
</thead>
<tbody>
<tr>
<td>glycine</td>
<td>Gly-Gly</td>
<td>cyclo(Gly-Gly)</td>
</tr>
<tr>
<td>alanine</td>
<td>Gly-Ala</td>
<td>cyclo(Gly-Pro)</td>
</tr>
<tr>
<td>β-alanine</td>
<td>Gly-Thr</td>
<td>cyclo(Leu-Gly)</td>
</tr>
<tr>
<td>α-aminoacetic acid</td>
<td>Pro-Gly</td>
<td></td>
</tr>
<tr>
<td>β-aminoacetic acid</td>
<td>Gly-Val</td>
<td></td>
</tr>
<tr>
<td>γ-aminoacetic acid</td>
<td>Val-Gly</td>
<td></td>
</tr>
<tr>
<td>aspatic acid</td>
<td>Gly-Glu</td>
<td></td>
</tr>
<tr>
<td>glutamic acid</td>
<td>Glu-Gly</td>
<td></td>
</tr>
<tr>
<td>valine</td>
<td>Leu-Gly</td>
<td></td>
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<tr>
<td>isovaline</td>
<td></td>
<td></td>
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<tr>
<td>isoleucine</td>
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</tbody>
</table>

[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.

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
certainty 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
indicating their abundances were minimally influenced by contamination with terrestrial 1-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 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,[26] which did not incorporate a condensing reagent. Peptides were undetectable in the H₂S 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 pKₐ value of the amino acid,[18] the reacting amino acid species would be H₃N⁺–\(\text{C(RR'-COO⁻)}\). As the pKₐ 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 1 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 NH₄Cl, 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...
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 glycineamide (pK_a ≈ 8)[30] 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 glycineamide is lower than that of the amino group of glycine, which is approximately 9.8.[18] As a consequence, the NH₂ group of glycineamide will be less protonated under such a regime, while the NH₃ group of glycine will be more protonated. Thus, glycineamide 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 glycineamide is about 3 days, while at pH = 7.95 and 75 °C, the half-life of glycineamide 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), glycineamide 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.

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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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).
Keywords: amino acids · cyanamide · mass spectrometry · peptides · polymerization