

PREBIOLOGICAL PROTEIN SYNTHESIS

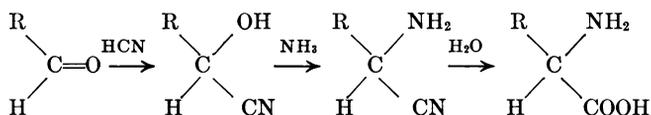
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A major concern of chemical evolution research¹⁻⁴ is to find an answer to the question: How were proteins originally formed on Earth before the appearance of life? A widely held view stimulated by the speculations of Oparin,⁵ Haldane,⁶ Bernal,⁷ and Urey⁸ is that the formation of polypeptides occurred via two essential steps, α -amino acid synthesis initiated by the action of natural high-energy sources on the components of a reducing atmosphere, followed by polycondensations in the oceans or on land. The results of a dozen years of simulation experiments¹⁻⁴ appear to support this view.

Experiments in which high-energy radiations were applied to reduced mixtures of gases have yielded many of the 20 α -amino acids commonly found in proteins. The pioneering research of Miller⁹ showed that glycine, alanine, aspartic acid, and glutamic acid were among the products obtained by passing electric discharges through a refluxing mixture of hydrogen, methane, ammonia, and water. Extensions of these studies by Abelson¹⁰ and others¹⁻⁴ showed that α -amino acid synthesis could be effected by almost any source of high energy so long as the starting mixture contained water and was reducing. Since mechanism studies by Miller⁹ indicated that aldehydes and hydrogen cyanide were transient intermediates during the course of the reaction, it was concluded that the α -amino acids were formed by the well-known Strecker route involving hydrolysis of aminoacetonitriles arising from the interactions of aldehydes, hydrogen cyanide, and ammonia.



No peptides were detected by Miller, but in similar experiments run at lower temperatures, Grossenbacher and Knight¹¹ showed the presence of several peptidic products as well as additional α -amino acids.

In a second type of investigation, Oró¹² and co-workers reacted hydrogen cyanide in aqueous ammonia and detected glycine, alanine, and aspartic acid. Since black HCN polymers¹³ were also present, it seemed reasonable to suggest¹² that the α -amino acids were formed by hydrolysis of HCN oligomers such as the tetramer, diaminomaleonitrile,¹⁴ and the trimer, aminomalononitrile.¹⁵ Lowe, Rees, and Markham¹⁶ extended these studies, obtaining at least five other α -amino acids as well as amino acid polymers believed to have been formed from the free acids. Purines including adenine¹² were also detected in the HCN reaction products.

A third type of research has shown that peptides and polypeptides can be directly synthesized from α -amino acids under certain conditions. In aqueous ammonia solutions at about 160°C, Oró and Guidry^{12, 17} found that up to 18 glycine molecules could be linked together. Glycine precursors such as aminoacetonitrile could be converted to small peptides in aqueous reactions¹² or by heating with clay.¹⁸ Condensing agents that may have been present in primordial times have also been

used. By allowing free α -amino acids to stand in aqueous solutions (acidic) containing cyanamide, Ponnamperna and Peterson¹⁹ and Steinman, Lemmon, and Calvin²⁰ obtained tripeptides, while in the absence of water, Schramm²¹ and co-workers obtained polypeptides by using phosphorus pentoxide in ether. In the simplest condensation method of all, Fox,²² Harada, and co-workers isolated polymers of high molecular weight (up to 300,000) after heating mixtures of α -amino acids to 180°C under anhydrous conditions, lower temperatures being permissible when polyphosphoric acid was used as a solvent.

From these three types of studies, then, it appears to be well established that α -amino acids could and would have been synthesized readily in the reducing environment of primitive Earth. Less certain are the assumptions concerning the polymerization step. The specialized conditions suggested by the model experiments do not have universal appeal²³ since anhydrous locales, high-temperature surface milieu, and acidic oceans or pools are not characteristic of a young, developing planet. Since the thermodynamic barrier to spontaneous α -amino acid polymerization is not easily overcome,²⁴ and indeed seems impassable by any reasonable condensation mechanism, a completely different sequence of events leading to polypeptide formation has been postulated by Kliss and Matthews.²⁵ In their view of chemical evolution, hydrogen cyanide formed by solar irradiation of a methane-ammonia atmosphere spontaneously dimerized to yield aminocyanomethylene,²⁶ $\text{H}_2\text{N}-\dot{\text{C}}=\text{C}=\dot{\text{N}}$, a ground state²⁷ 1,3-biradical.²⁸ Addition polymerization of this bifunctional intermediate led to the formation in the atmosphere of polymeric peptide precursors which became converted to polypeptides as they settled in the primeval oceans. In the following discussion, we present evidence in favor of this hypothesis, describing experiments in which peptides were synthesized directly from hydrogen cyanide reactions without any intervening formation of α -amino acids.

Experiments and Results.—Two types of experiments were performed, deliberately related to the high-energy radiation reactions and the hydrogen cyanide studies outlined above. In one type, methane and ammonia were subjected to an electric discharge while, in the other, hydrogen cyanide was allowed to interact with anhydrous ammonia. In accord with the proposed model, water was rigorously excluded as a reactant in all experiments in order to simplify the reaction mixture, maximize the possibility of vapor phase polymerization, and demonstrate that oxygenated species such as aldehydes are not essential synthetic intermediates. Further, in working up reaction products, a two-step hydrolysis procedure was devised to enable the presence of any peptides to be detected. In the first step, mild hydrolysis conditions were used to convert any imino groups to carbonyl groups and yet not cleave peptide bonds. In the second step, strong hydrolysis was applied in order to convert peptides, if present, to identifiable α -amino acids.

Methane-ammonia reactions: In an electric discharge apparatus similar in principle to the reactors used by Miller and others, gas mixtures were continuously circulated by thermal diffusion so that volatile reaction products passed repeatedly through the spark between two tungsten electrodes. In a typical run conducted in a 5-liter capacity apparatus, the reaction chamber was filled to a pressure of 500 mm with a 1:1 (v/v) mixture of methane (Matheson research grade, 99.99% purity) and ammonia (Matheson, anhydrous) that had been passed through a column of

anhydrous sodium hydroxide pellets. The spark was ignited (Jefferson transformer no. 721-111, 15,000 v), causing slight warming in the electrode region of the chamber which otherwise was maintained at room temperature. The first observed products from methane-ammonia breakdown were yellow to brown droplets on the walls near the spark. Volatile compounds detected during the reaction consisted mainly of hydrogen, nitrogen, and hydrogen cyanide, with smaller amounts of methane, ethane, ethylene, and acetylene.²⁹ Nonvolatile reaction products were allowed to accumulate for up to 60 hr when nearly all of the methane had been consumed. By this time, the oil had taken on the appearance of a black viscous tar, and the inside wall of the chamber had become coated with a brown film.

The residues were washed out into 50 ml of cold 0.1 *N* HCl and heated at 100°C for 30 min. The resulting mixture was divided into two fractions (A and B), both consisting of black solids and a dark solution. The concentration of acid in fraction A was brought to 6 *N* by the addition of hydrochloric acid, and the mixture was heated to 100°C for 24 hr to effect complete hydrolysis of any peptide-like materials. The hydrolysate was evaporated to dryness *in vacuo* at 70°C. α -Amino acid products were isolated by two-step ion-exchange chromatography^{30, 31} and then characterized by a Beckman amino acid analyzer (model 120B). Lysine, histidine, aspartic acid, threonine, serine, glycine, alanine, and isoleucine were positively identified (Table 1, column 1).

Three methods of analysis were used to obtain evidence for the presence of peptides in fraction B, viz., thin-layer chromatographic (TLC) separations followed by ninhydrin³² treatment, analysis by a Technicon AutoAnalyzer,³³ and dinitrophenylation (DNP)^{24, 35} experiments. Fraction B was first partially purified by treatment with acid-washed decolorizing charcoal which was independently found not to alter the composition of dilute acid solutions of several α -amino acids and peptides. TLC analysis of the resulting filtrate showed five ninhydrin-active spots, their colors suggesting the presence of peptides rather than α -amino acids. The

TABLE 1
 α -AMINO ACID CONTENT OF HYDROLYSIS RESIDUES
(BECKMAN AMINO ACID ANALYZER)

| Component found* | CH ₃ -NH ₂ Reaction | | | HCN-NH ₂ reaction 10 ² (μ moles) † |
|------------------|---|---------------------------------------|-------|--|
| | 1 | 2 10 ² (μ moles) † | 3 | |
| Basic | | | | |
| Lys | 13 | 1 | 2 | Trace |
| His | 13 | 0.4 | 3 | 16 |
| NH ₂ | 85 | 1830 | 2400 | 162 |
| Unknown | — | Trace | Trace | Trace |
| Neutral | | | | |
| Unknown | Trace | Trace | Trace | Trace |
| Asp | 24 | 3 | 28 | 30 |
| Unknown | Trace | Trace | — | — |
| Thr | 15 | 3 | 1 | 17 |
| Ser | 22 | 2 | 3 | 14 |
| Unknown (2) | Trace | — | † | — |
| Gly | 589 | 130 | 390 | 165 |
| Ala | 5 | 1 | 26 | — |
| Unknown | Trace | Trace | Trace | — |
| Ileu | 6 | 1 | — | 0.6 |

* In order of elution from columns. Several exceedingly weak peaks have been omitted from this table.

† Quantities of components should be compared only within single columns, not between columns, since the method of purification influenced ammonium salt content of the samples.

‡ Glutamic acid was detected.

filtrate was freeze-dried. A portion analyzed by the Technicon AutoAnalyzer contained at least six possible peptidic components. Strong hydrolysis of a second portion of the freeze-dried residue in 6 *N* HCl at 100°C for 24 hr and evaporation of the solvent *in vacuo* gave a white solid which was mechanically analyzed for α -amino acids. Essentially the same α -amino acids were obtained (Table 1, column 2) as in the hydrolysis of the crude reaction product. A third portion of the peptidic residue was treated with fluorodinitrobenzene (FDNB), the derivatives then being separated into three fractions by selective solvent extraction. If α -amino acids and peptides had been present, DNP-amino acids would have been collected into ether, DNP-derivatives of small peptides and basic amino acids into *n*-butanol-ethyl acetate (1:1, v/v), and DNP-derivatives of larger peptides into the aqueous phase. Portions of each of the three DNP-derivative solutions were evaporated to dryness, and the residues were hydrolyzed in 6 *N* HCl at 100°C for 24 hr. TLC analysis of the hydrolysates of the ethereal and butanol-ethyl acetate fractions indicated that no α -amino acids had formed upon hydrolysis. By contrast, the developed chromatogram of the hydrolysate of the aqueous fraction showed a very intense band of ninhydrin-active components, with glycine as the major component of the mixture. Treatment of this hydrolysate with FDNB followed by TLC analysis of the derivatives allowed the positive identification of DNP-glycine, -isoleucine, -serine, and -aspartic acid in the mixture.

Further evidence for peptide formation was obtained from another spark reaction by working up the brown-black products under conditions resembling those postulated for primitive Earth. Instead of employing acid hydrolysis, distilled water was added to the material which was then allowed to stand at 25°C for a week, during which the pH of the suspension changed from 8.8 to 8.5. No α -amino acids were present. After strong hydrolysis of a portion of the dark brown suspension, several α -amino acids were detected, glycine being most abundant. Filtering the remainder of the suspension gave a brown solution which was freeze-dried to yield a pale brown solid. This was fractionated by Sephadex gel filtration using 0.1 *N* ammonium acetate as the eluent. Fractions were collected automatically and the optical densities at 210 μ were plotted against the effluent volume. Four peaks were most prominent and the fractions making up each peak were combined and freeze-dried to give a yellow intractable tar and three samples of yellow fluffy solids which must have different molecular weights since separation by gel filtration is based on size differentiation. The solids show infrared bands which can be assigned to amino (3390 cm^{-1}), cyano (2350 and 2400 cm^{-1}), and carbonyl or imino (1790 cm^{-1}) groups. Technicon AutoAnalyzer results indicate that each of the three solids consist of several peptides. After strong hydrolysis, glycine and seven other α -amino acids were detected (Table 1, column 3). Rigorous characterization of these materials believed to be peptides is in progress.

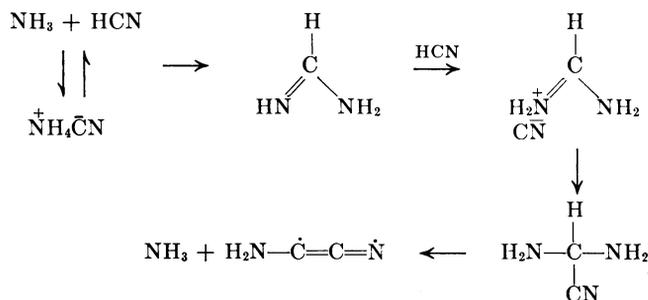
Hydrogen cyanide-ammonia reactions: In studies of hydrogen cyanide reactions catalyzed by dry ammonia, anhydrous HCN was allowed to stand over crystalline ammonium cyanide in a closed glass tube. In a typical experiment, a mixture of anhydrous HCN and liquid ammonia (10:1, v/v) frozen in a liquid nitrogen bath was allowed to warm up slowly and was then held at room temperature for 48 hr. By this time, the initially colorless mixture had been completely replaced by a black solid and some white needles; these were separated by ether extraction (Soxh-

let) and shown to be diaminomaleonitrile, mp 182–184°C. Ether-insoluble black polymer recovered from the extractor was mixed thoroughly with 1.0 *N* HCl. The resultant mixture of a deep red solution and a black tarry solid was purified by ion-exchange chromatography.³⁰ TLC analysis (Eastman fluorescent plates) showed the presence of adenine, made visible by treatment with Hg⁺⁺-eosin reagent³⁶ and by blocking of the plate fluorescence under 3660 Å light.³⁰ Similar techniques applied to the crude product of the methane-ammonia reaction did not show the presence of adenine, although a possible precursor, 4(5)-amino-5(4)-cyanoimidazole,^{12, 25} was detected by its ultraviolet spectrum.³⁰

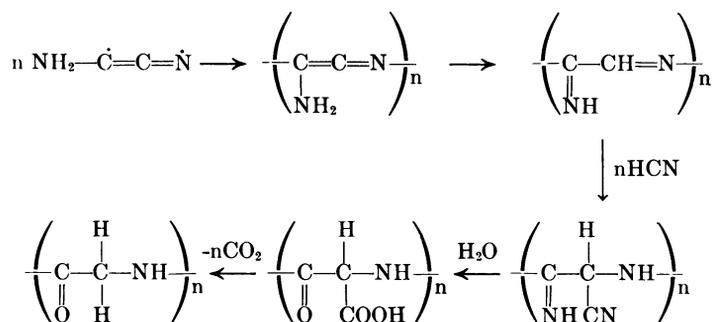
Peptide and α -amino acid analyses were carried out on samples of the black polymeric product using the methods already described. Peptide-like materials were detected after mild hydrolysis, while under more rigorous conditions essentially the same mixture of α -amino acids was shown to be present, glycine being the main constituent (Table 1, column 4).

Discussion.—We conclude that in these two types of reactions carried out in the absence of water, polymeric peptide precursors as well as intractable black polymers were formed by polymerization reactions of hydrogen cyanide catalyzed by ammonia. Mild hydrolysis then brought about conversion to peptides consisting mainly of glycine with at least seven other α -amino acid residues incorporated within the polymers. Adenine was formed when the concentration of HCN was sufficient.³⁷ It seems probable to us that most of the simulation reactions previously carried out under aqueous conditions must have proceeded in the same way, through spontaneous peptide formation from HCN followed by hydrolytic breakdown to α -amino acids. In our opinion, prebiological Strecker synthesis was highly unlikely, not only because we have shown that there is no need to invoke reactions of aldehydes but also because we have observed that aminoacetonitrile in water or weak ammonia solutions is quite unstable. Black polymers form gradually, more rapidly when heated,^{12, 17, 18} suggesting ready breakdown to hydrogen cyanide. On further hydrolyzing the products a mixture of α -amino acids is obtained. Glycine is the sole α -amino acid product only when aminoacetonitrile is hydrolyzed by strong acids under the usual Strecker conditions.

To rationalize our experimental results, we propose the following mechanisms³⁸ to account for the synthesis of polypeptides by HCN polymerization reactions. First, with excess of hydrogen cyanide in the presence of ammonia, aminocyanomethylene was formed catalytically by way of the labile intermediates formamidine and diaminoacetonitrile.^{39, 40} Biradical polymerization then took place, proceeding rapidly by a geometric rather than an arithmetic progression. Tautomerism of the



resulting polyaminoketenimine followed by saturation with HCN yielded a stereoregular²⁵ helical polymer, a protopolycyanide which can formally be regarded as polyaminomalnonitrile.⁴¹ On settling into water, gradual hydrolysis of imino and cyano groups occurred to yield polyaminomalonic acid which eventually was converted to polyglycine by decarboxylation.⁴²



The formation of polypeptides with side groups other than hydrogen along the chain was initiated before the meeting with water. Some of the activated nitrile groups projecting from the protopolycyanide helices were attacked by HCN to give iminocyano groups which could then react further with other HCN molecules. It seems likely that most of the 20 side chains found in proteins originated by HCN addition reactions of this type, followed by reactions with water. The other side chains containing aromatic rings or sulfur atoms would have been formed through further interactions involving acetylene and hydrogen sulfide, two expected components of the primitive atmosphere.

Four aeons or so ago this over-all polymerization sequence⁴³ proceeded efficiently and selectively in the atmosphere of Earth and led to the formation of a protein-dominated layer that concentrated in the oceans of the then sterile planet. Today it is likely that such processes still are occurring on Jupiter and on the myriad other planets⁴⁴ possessing atmospheres of methane and ammonia, cosmic molecular precursors of hydrogen cyanide and life.

Summary.—Polymeric peptide precursors were synthesized spontaneously in the absence of water by polymerization reactions of hydrogen cyanide catalyzed by ammonia. Similar products were obtained by applying electric discharges to dry mixtures of methane and ammonia. The polymers were converted to peptides by mild hydrolysis and to a mixture of at least eight of the common α -amino acids by strong hydrolysis. Adenine or adenine precursors were also detected in the anhydrous products. Following the chemical evolution model of Kliss and Matthews, these results show that proteins could have evolved readily in the reducing environment of primitive Earth and other planets without the intervening formation of α -amino acids.

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peptides in the primitive oceans (see ref. 5, p. 213). As early as 1875, E. Pfüger (see ref. 5, p. 84) considered that "protein" was formed from cyanogen radicals.

³⁹ M. Seefelder [*Angew. Chem. Intl. Ed.*, **5**, 321 (1966)] has described some analogous reactions of HCN with amines.

⁴⁰ The formation of formamidine from ammonium cyanide is analogous to the historic Wöhler reaction by which urea was formed from ammonium cyanate. Ready stabilization of formamidine by the addition of HCN would yield formamidinium cyanide, which, like all organic cyanides, would tend to assume a covalent nitrile form. The resulting diaminoacetonitrile would readily eliminate ammonia and generate aminocyanomethylene.

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⁴³ The direct photochemical dimerization and polymerization of HCN is another possibility²⁵ we are exploring.

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