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HCN: A Plausible Source of Purines, Pyrimidines and Amino Acids on the Primitive Earth*

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Summary. Dilute (0.1 *M*) solutions of HCN condense to oligomers at pH 9.2. Hydrolysis of these oligomers yields 4,5-dihydroxypyrimidine, orotic acid, 5-hydroxyuracil, adenine, 4-aminoimidazole-5-carboxamide and amino acids. These results, together with the earlier data, demonstrate that the three main classes of nitrogen-containing biomolecules, purines, pyrimidines and amino acids may have originated from HCN on the primitive earth. The observation of orotic acid and 4-aminoimidazole-5-carboxamide suggests that the contemporary biosynthetic pathways for nucleotides may have evolved from the compounds released on hydrolysis of HCN oligomers.

Key words: HCN – Cyanide – HCN oligomers – 4,5-Dihydroxypyrimidine – Orotic Acid – 5-Hydroxyuracil – Adenine – 4-Aminoimidazole-5-carboxamide – Prebiotic – Primitive earth

Introduction

HCN is considered to have been an important source of biological molecules on the primitive earth. Oro and Kimball (1961, 1962) first demonstrated the syntheses of the purine adenine (*6*) and the amino acids glycine, alanine and aspartic acid from HCN. These results have been confirmed and extended by several groups and it is now known that adenine, 4-aminoimidazole-5-carboxamide (*4*), hydantoins and at least seven amino acids are formed with HCN as the only reactant. (Oro and Kamat, 1961; Lowe et al., 1967; Matthews and Moser, 1967; Ferris et al., 1973a; Ferris et al., 1974a, 1974b; Labadie et al., 1967, 1968).

Aqueous, pH 9.2 solutions of cyanide of concentration greater than 0.01 *M* condense to give a tetramer (*2*) which reacts further to give oligomers (Sanchez et al., 1967). These oligomers are compounds of unknown structure of molecular weight

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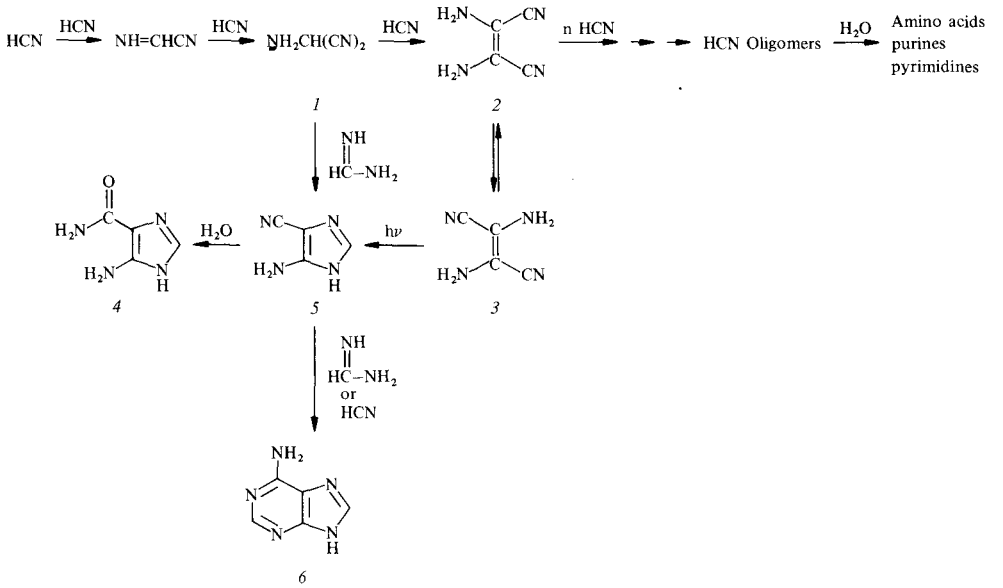
500–1000 which yield biological molecules such as amino acids and purines upon hydrolysis (Scheme 1) (Ferris et al., 1972, 1973b). Similar solutions in which the cyanide concentrations are 0.01 *M* or less do not form oligomers but instead the cyanide hydrolyzes in a stepwise fashion to yield formamide and formic acid. Oligomer formation does take place in preference to hydrolysis when 0.01 *M* solutions of cyanide are cooled to -23.4°C. A eutectic containing 74.5 mole per cent cyanide is formed at this temperature. The HCN concentration is so great that oligomer formation proceeds at an appreciable rate even though the temperature is -23.4°C (Sanchez et al., 1966).

In Oro's initial investigations (1961, 1962), adenine was found as a reaction product when 1–11 *M* solutions of HCN were used. Indirect evidence suggested that this synthesis proceeds via the condensation of formamidine with a trimer of HCN (aminomalononitrile, *1*) and with 4-aminoimidazole-5-carbonitrile (*5*) (Scheme 1) (Sanchez et al., 1967; Ferris and Orgel, 1965, 1966a,b). This synthesis would not be plausible in dilute solution because of the competing hydrolysis of formamidine to formamide (Sanchez et al., 1967). Previously we found that diaminomaleonitrile undergoes a very efficient photochemical rearrangement to 4-aminoimidazole-5-carbonitrile, a compound which readily yields a wide variety of purines under conditions which may have existed on the primitive earth (Ferris and Orgel, 1966a,b; Ferris et al., 1969; Ferris and Kuder, 1970; Sanchez et al., 1968). The latter is a more plausible purine synthesis since it does not require a high steady state concentration of formamidine.

Scheme 1.



Experimental

General Procedures. UV spectra were determined on a Unicam SP 800 and IR spectra were determined on a Perkin-Elmer Model 137 spectrophotometer. Paper chromatography was performed by the ascending technique for 18–24 h using Whatman 3MM paper and the following solvent systems (1) 1-propanol/NH_4OH (17%) (3:1); (2) 1-butanol/acetic acid/H_2O (12:3:5); (3) *t*-butyl alcohol/methyl ethyl ketone/formic acid/H_2O (40:30:15:15); (4) 1-butanol/water (86:14); (5) isopropyl alcohol/H_2O/conc. HCl (56:18.4:16.6); (6) 1-butanol/acetic acid/water (2:1:1); (7) isopropyl alcohol/NH_4OH/H_2O (20:1:2); (8) benzene/acetic acid/H_2O (125:72:3); (9) 1-butanol/acetic acid/water (4:1:1); (10) ethyl acetate/formic acid/H_2O (70:20:10); (11) ethyl acetate/formic acid/H_2O (60:5:35 – upper layer); (12) t-butyl alcohol/methyl ethyl ketone/H_2O/formic acid (44:44:11:0.26). The Dowex 1-X8 (200 g) was washed batchwise before use with 4 ℓ of distilled water then packed in a glass column and washed with 1 ℓ of distilled water, 1 ℓ of 4*N* HCl, distilled water, 1 ℓ of 4*N* NH_4OH and finally with distilled water. The Dowex 50W-X8 (200 g) was washed in the same way except the 4*N* NH_4OH wash preceeded the 4*N* HCl wash.

Gas Chromatography and Combination GC/MS. Gas chromatographic procedures similar to those of Butts (1972) were used. Gas chromatography was performed on 6 ft. 0.2 mm i.d. glass column packed with either 3% OV-1 or 3% OV-17 on 80/100 mesh Chromosorb W-HP. The column temperature was maintained initially at 70° or 90° for 3 or 4 min and was then programmed to 275° at 10°/min. The injection port was maintained at 240° and the flame detector at 300°. The gas flow rates were: helium or nitrogen, 40 ml/min; hydrogen, 30 ml/min; and air, 300 ml/min. Standard samples of n-alkanes were run under the same conditions to give retention times relative to the internal standards, expressed as retention indices. The analyses were performed on either a Varian 2400 or Hewlett-Packard 7620A chromatograph equipped with flame ionization detectors. The GC/MS analyses were performed on a Perkin-Elmer 990 gas chromatograph interfaced with the ion source of a CEC 21-491 mass spectrometer. The samples passed through a membrane separator from the gas chromatograph to the mass spectrometer (Folsome et al., 1973). Data processing was performed on a Decision GC/MS computer system.

Samples were converted to volatile trimethylsilyl derivatives for gas chromatography unless noted otherwise. The samples (0.5–4 mg) were dissolved in 50 μl of pyridine and to this was added 50 μl of Regisil ™ which is a mixture of 99% bis(trimethylsilyl)trifluoroacetamide and 1% trimethylchlorosilane. The samples were sonicated to facilitate solution, sealed in ampuls and heated at 150° for 2.5 h or at 60° for 18–24 h. The cooled reaction solution was injected directly into the gas chromatograph.

Oligomerization and Fractionation of HCN. A 0.1 *M* solution of HCN was adjusted to pH 9.2 by the addition of conc. NH_4OH and the solution was stored in the dark in glass-stoppered bottles at room temperature for 4–12 months. The concentration of cyanide as measured by the method of Scoggins (1972), decreased by about one half after six months reaction time. The pH of the solution was still 9.2 when the oligomerization was terminated. One liter of the oligomerization mixture was filtered to

remove a small amount of black insolubles and it was then added to a column of 200 g of Dowex 1 (OH^-). The column was eluted with 2 ℓ of distilled water and the combined eluates were then chromatographed on 200 g of Dowex 50 (H^+). The Dowex 50 column was eluted with 1–2 ℓ of distilled water and the eluate was concentrated using a rotary evaporator to give the neutral fraction. The Dowex-1 column was then washed with 3–4 ℓ of 3 *N* HCl and the eluate was concentrated to yield 430 mg of acidic and amphoteric oligomers. This fraction was heated at 70° in vacuo for 7–10 days to sublime the oxalic acid and NH_4Cl present leaving 325 mg of acidic and amphoteric oligomers. The Dowex 50 column was washed with 2 ℓ of 3 *N* NH_4OH and concentrated to give 25 mg of basic material.

To prepare HCN oligomers in the absence of oxygen, the 0.1 *M* cyanide solution was adjusted to pH 9.2 with NH_4OH. This solution was cooled in a liquid nitrogen bath and subjected to four freeze-pump-thaw cycles. Nitrogen gas was admitted to the flask on the last cycle. The flask was maintained in an atmosphere of nitrogen for 7 months before the oligomerization was terminated and the products were fractionated by ion exchange as described above. The yield of acidic and amphoteric oligomers before sublimation was 584 mg per liter of 0.1 *M* cyanide.

Hydrolysis and Fractionation of the Hydrolysate of the HCN Oligomers. The oligomer hydrolyses were performed for 24 h at 110° in sealed ampuls in either 6 *N* HCl or in aqueous solution which was adjusted to pH 8.5 with conc. NaOH. A final pH of 8.3–8.5 was observed after the pH 8.5 hydrolyses were terminated. The hydrolysate solutions were concentrated to dryness using a rotary evaporator and then dissolved in distilled water for analysis. Oligomers prepared in the absence of oxygen were degassed by 4 freeze-pump-thaw cycles after dissolution in 6 *N* HCl or at pH 8.5 before hydrolysis at 110°.

About 10 ml of an aqueous solution of the hydrolysate was passed through a column of 25–30 g of Dowex 50 (H^+) and the acidic and neutral fraction was eluted with water. The basic and amphoteric compounds were eluted with 4 *N* NH_4OH.

GC/MS Analysis of the HCN Oligomer Hydrolysate. The basic and amphoteric fraction of the 6 *N* HCl hydrolysate (Chart 2) was silylated and chromatographed on an OV-1 column. The presence of the following compounds was established by the comparison of their mass spectrum with that of a silylated authentic sample: Glycine, 4,5-dihydroxypyrimidine, *meso*- and *dl*-diaminosuccinic acid and β-alanine (Fig.1). The relative yields of the silylated derivatives of glycine and 4,5-dihydroxypyrimidine were calculated to be 1:0.85 respectively from their relative ion monitor responses on the mass spectrometer. Several unidentified compounds were also observed.

The GC trace of the pH 8.5 hydrolysate was similar to that of the 6 *N* HCl hydrolysate with the exception that no 4,5-dihydroxypyrimidine was observed and lower yields of the other biomolecules were found when the same hydrolysis time period was used. The presence of glycine and the *meso*- and *dl*-diaminosuccinic acid was confirmed from the mass spectra. No significant differences were observed in the nature of the biomolecules formed from oligomers prepared in the absence or presence of oxygen.

The basic and amphoteric fraction resulting from the acid hydrolysis of the basic HCN oligomers (Chart 1) contained α-aminoisobutyric acid, glycine, 4,5-dihydroxypyrimidine and β-alanine as shown by the GC/MS analysis of the silylated sample.

Chart 1.

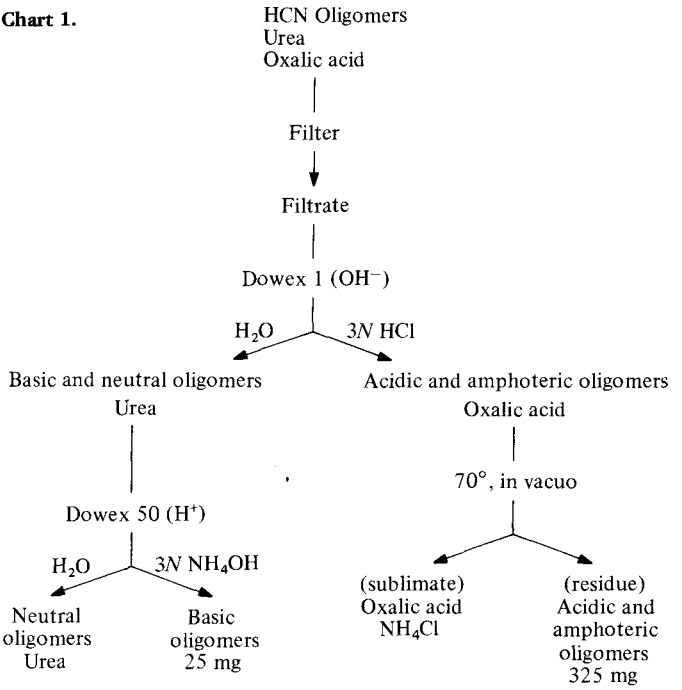
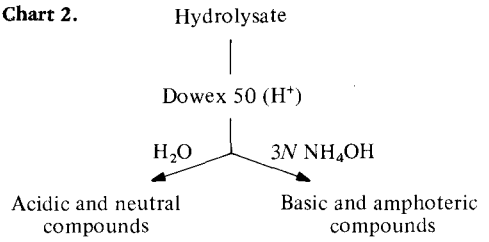


Chart 2.



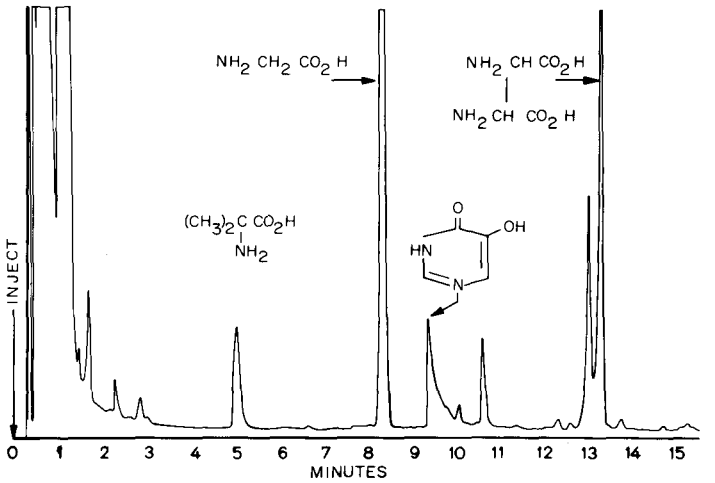


Fig. 1. Gas chromatographic trace of the trimethylsilyl derivatives of the basic and amphoteric compounds formed on acid hydrolysis of the HCN oligomers

The silylated acid and neutral fraction (Chart 2) obtained by hydrolysis of the acidic and amphoteric oligomers (Chart 1) with 6 *N* HCl or at pH 8.5 did not give reproducible GC/MS results. Apparently the silyl derivatives decomposed after elution from the GC column but before reaching the ion source of the mass spectrometer. Oxalic acid was consistently observed as the major hydrolysis product. The presence of 5-hydroxyuracil and orotic acid was suggested by one GC/MS run but this could not be reproduced. The presence of these compounds was confirmed after the substances were purified as described later.

4,5- Dihydroxypyrimidine. This substance is a major product in the basic and amphoteric fraction of the acid hydrolysate (Chart 2). It gives an intense UV-absorbing spot on paper chromatography of this fraction which gives an orange color with diazotized sulfanilic acid (Grimmett and Richards, 1975). This compound was not observed when the hydrolysis was performed at pH 8.5 but another pyrimdine is formed which is converted to 4,5-dihydroxypyrimidine on acid hydrolysis. The 4,5-dihydroxypyrimidine exhibits the following R_f values: system (R_f); (1) 0.25; (2) 0.52; (4) 0.35; (7) 0.12; (8) 0.68. The following UV maxima were observed: λ_{max} 255 nm (0.1 *M* HCl) 260 and 287 nm (0.1 *M* NaOH) and 268 nm (distilled water). It exhibited a retention time of 9.75 min (retention index = 13.70) on OV-1 and 9.45 min (retention index = 14.54) on OV-17. The quantitative yield of 4,5-dihydroxypyrimidine in the acid hydrolysate of the HCN oligomers was found to be 17.5 mg per liter of the oligomerization mixture.

Combination GC/MS of the silylated derivative gave a molecular ion at m/e 256 and an M-15 at m/e 241. High resolution GC/MS, performed in the laboratory of Dr. A. Burlingame (Space Sciences Laboratory, University of California at Berkeley) gave molecular weights of 256.1057 and 241.0832 respectively for these peaks. The molecular formula calculated for $C_{10}H_{20}N_2O_2Si_2$ is 256.1063 and for $C_9H_{17}N_2O_2Si_2$ is 241.0829. These data require an empirical formula of $C_4H_4N_2O_2$ for the parent compound.

An authentic sample of 4,5-dihydroxypyrimidine was prepared by the acid hydrolysis of 4,5-diaminopyrimidine (Aldrich) using a procedure similar to that of McOmie and Turner (1963): mp about 274° (start dec. at 265°) lit mp 268-269° decomp. (McOmie and Turner, 1963) λ_{max} (1 *N* HCl) 255 nm, ϵ 9180; 1 *N* NaOH 260 nm, ϵ 7620 amd 287 nm, ϵ 7060). The synthetic sample exhibited UV and mass spectral data identical with those of the compound isolated from the HCN oligomers.

Orotic Acid. Orotic acid was detected in the acid and amphoteric fraction of the oligomer hydrolysate (Chart 2) if the hydrolysis was performed at pH 8.5 or with 6 *N* HCl followed by hydrolysis with 1 *N* NaOH. No orotic acid was detected if the oligomers were not hydrolyzed or if only 6 *N* HCl was used for hydrolysis. The presence of orotic acid was established using the following methods:

a) Specific Color Test. A color test (Adachi et al., 1963; Kesner et al., 1975) was used to establish the presence of orotic acid in the total hydrolysate before it was fractionated on an ion exchange resin. The results are given in Table 1. The yields were determined by comparison with standard orotic acid solutions measured at 480 nm. The absorbance at 480 nm was corrected with a blank containing the colorimetric reagents but not orotic acid. It was determined by paper chromatography that barbituric acid, a substance which gives the same color test with this reagent, was not present in the hydrolysate.

Table 1. Orotic acid yield (μg)[a]

	Acid/Base Hydrolysis	*pH 8.5 Hydrolysis*
100 ml of oligomerization mixture	5.5	nd
50 mg of acidic and amphoteric oligomers	8.3[b]	43
20 mg of acidic and amphoteric oligomers prepared in the absence of oxygen	nd	10[c]

[a]Yields determined colorimetrically as described in the text, nd = not determined
[b]This yield is equivalent to 5.4 μg per 100 ml of the oligomerization mixture since this volume contains 30–35 mg of acidic and amphoteric oligomers
[c]This yield corresponds to 45 μg of orotic acid per 50 mg of the oligomers prepared in the presence of oxygen. (584 mg/ℓ of acidic and amphoteric oligomers are obtained in the absence of oxygen as compared to 325 mg/ℓ in the presence of oxygen). This corresponds to a total yield of 280 μg/ℓ orotic acid from the oligomerization mixture

b) Paper Chromatography. Orotic acid was observed as a UV-absorbing substance which had the same R_f as an authentic sample in systems 1, 3, 5, and 6. This UV-absorbing area gave a positive color test for orotic acid (section a). Orotic acid was also detected by paper chromatography and ultraviolet spectroscopy in the hydrolysate of the oligomers prepared in the absence of oxygen.

c) Gas Chromatography/Mass Spectrometry. A sample of the acid and neutral hydrolysate (Chart 2) from the acidic and amphoteric oligomers (Chart 1) prepared by pH 8.5 hydrolysis was partially purified by paper chromatography in system 1. The UV absorbing area with the same R_f as orotic acid was eluted, silylated and subjected to gas chromatography on OV-1 and OV-17. The predominant peak had the same retention time as orotic acid and exhibited the same mass spectrum as an authentic sample of silylated orotic acid.

5-Hydroxyuracil. The acidic and neutral fraction (Chart 2) from the acid hydrolysis of the acidic and amphoteric oligomers (Chart 1) was chromatographed in system 10 and the UV-absorbing material with the same R_f as 5-hydroxyuracil was eluted and rechromatographed in system 2. The UV spectra of the authentic 5-hydroxyuracil and the material from the HCN oligomers were identical when measured in acidic and neutral solutions (λ_{max} 275 nm) and were similar in basic medium (λ_{max} 298 nm and 295 nm respectively). The silyl derivative of the substance isolated from the HCN oligomers gave the same gas chromatographic retention time on OV-1 and OV-17 and the same mass spectrum as a standard sample. The mass spectrum was identical with a published spectrum. (Markey et al., 1974). The yield of 5-hydroxyuracil, estimated by comparison of the intensity of the UV absorption on the paper chromatogram with varying concentrations of standards applied to the same paper, was 80-120 μg/l of the total oligomerization mixture.

Unidentified Pyrimidine Released by pH 8.5 Hydrolysis of HCN Oligomers. Chromatography in system 1 of the basic and amphoteric fraction (CHart 2) of the pH 8.5 hydrolysate revealed a large array of UV-absorbing and fluorescent spots, several of which gave a blue color when sprayed with the Pauly Reagent (diazotized sulfanilic acid) (Grimmett and Richards, 1965). The substance which exhibited an R_f value of 0.1-0.2 in system 1 as well as UV absorption and a blue Pauly color was eluted and rechromatographed in system 10. This material exhibited an R_f of 0.1, it yielded 4,5-dihydroxypyrimidine on acid hydrolysis and it exhibited the following UV maxima: 280 nm (aqueous solution and 1 *N* HCl) and 290 nm (1 *N* NaOH). This compound is insoluble in the silylation mixture and as a consequence it has not been possible to obtain mass spectral data. Our observation of a UV spectrum, a positive Pauly test and its hydrolysis to 4,5-dihydroxypyrimidine constitutes strong evidence for the presence of a 4,5-substituted pyrimidine ring system in the compound.

Attempted Detection of 4-Hydroxypyrimidine. The detection of 4-hydroxypyrimidine in the Murchison meteorite (Folsome et al., 1973), prompted our search for it among the pyrimidines formed by hydrolysis of the HCN oligomers. It could not be detected in the basic and amphoteric fraction of the acid hydrolysate (Chart 2) when it was chromatographed in system 1. The UV absorbing material with the same R_f as 4-hydroxypyrimidine from this chromatogram was eluted and rechromatographed in system 10. No UV absorbing spot with the same R_f as 4-hydroxypyrimidine was observed within the limit of detection of 2.5-5 μg.

Adenine. Adenine was detected in the basic and amphoteric fraction of the hydrolysate (Chart 2) by comparison of the R_f of a UV absorbing substance in systems 1 through 5 with that of an authentic sample. This substance also gave a positive test for adenine with the silver nitrate - bromophenol blue reagent (Hais and Macek, 1963) after chromatography in system 1. The UV-absorbing area corresponding in R_f to adenine in system 1 exhibited the same UV spectrum as that of an authentic sample when measured in 0.1 *M* HCl (262.5 nm), 0.1 *M* NaOH (268.5 nm) and distilled water (261 nm). Optimal UV spectral data were obtained when the paper chromatographic sheets were washed with 1% oxalic acid solutions prior to use (Hanes and Isherwood, 1949). The adenine yield was 1 mg/l of the original oligomerization mixture. Adenine was also released by acid hydrolysis and pH 8.5 hydrolysis from oligomers prepared in the absence of oxygen.

The identity of adenine in the acid hydrolysate was confirmed by silylation of a sample which had been purified by chromatography in system 1 followed by elution from Dowex 1 with water. The major product exhibited the same retention time as an authentic sample when the silylated derivative was chromatographed on OV-1 and OV-17 (retention indices of 18.47 and 20.61 respectively)

Adenine Analysis Before Oligomer Hydrolysis. A 15 mg sample of the basic HCN oligomers (Chart 1) was chromatographed in system 1 with an authentic sample of adenine. Although no well-defined UV absorbing spot was obtained in the region which had the same R_f as authentic adenine this region was eluted with water and rechromatographed, in system 10. No spot corresponding to adenine was detected.

The limit of detection of adenine is 3-5μg. Since 15 mg of the basic oligomers corresponds to 0.5 l of the oligomerization mixture this means there is less than 6-10 μg of adenine in 1 l of the oligomerization mixture. Adenine was detected in the same sample after acid hydrolysis by the comparison of the R_f value (system 1) and the UV spectrum of an eluted sample with that of an authentic sample.

4-Aminoimidazole-5-Carboxamide. The basic and amphoteric hydrolysate (Chart 2) obtained by the acid hydrolysis of the acidic and amphoteric oligomers (Chart 1) was chromatographed using systems 1, 4 and 9. Several compounds which gave a positive test with diazotized sulfanilic acid (Grimmett and Richards, 1965) were observed. One of these was a faint spot which gave the same blue color and R_f value as an authentic sample of 4-aminoimidazole-5-carboxamide (AICA). A portion of the acid hydrolysate was chromatographed in system 1 by developing the paper twice. The paper was dried between developments. Elution of the material with the same R_f as AICA yielded a substance which exhibited a UV spectrum which was identical with that of AICA; λ_{max} 266.5 nm (0.1 *M* HCl), 277 nm (0.1 *M* NaOH) and 266 nm (water).

No AICA was detected initially when the corresponding oligomer fraction prepared in the absence of oxygen was hydrolyzed with 6 *N* HCl or at pH 8.5 for 24 h. Control experiments established that 2 mg of AICA is destroyed under these hydrolysis conditions. AICA was detected after a 1.5 h hydrolysis by its R_f value and color test using system 1. The substance produced by acid hydrolysis when eluted from the paper chromatogram gave the same UV spectrum as an authentic sample when measured in acidic, basic and neutral solution.

Hydrolysis of HCN Oligomers Without Prior Ion Exchange Fractionation. Since the possibility existed that the biomolecules released on hydrolysis of the HCN oligomers resulted from transformations taking place in the presence of the ion exchange resins, qualitative analyses were performed to see if the same compounds were released on direct hydrolysis of the oligomerization mixture. Amino acids, adenine, 4,5-dihydroxypyrimidine, AICA and orotic acid were all detected as hydrolysis products.

Biomolecules from HCN Oligomers Prepared from 1 M Cyanide Without Added NH_3. The pH of a 1 *N* NaCN solution was adjusted to 9.2 with conc. HCl and the solution was allowed to stand in a stoppered vessel in the dark for 1 year. The amphoteric and acidic HCN oligomers were isolated in the usual way (Chart 1). Acid hydrolysis of this fraction yielded adenine and 4,5-dihydroxypyridine. These substances were purified by paper chromatography as described previously. The adenine was identified by comparison of its UV spectrum in acidic and basic solution with that of an authentic sample. The 4,5-dihydroxypyrimidine was identified by comparison of the color produced with diazotized sulfanilic acid and diazotized p-nitroaniline (McOmie and Turner, 1965) with those colors produced using an authentic sample chromatographed on the same paper.

Guanidine. Paper chromatographic analysis of the basic and amphoteric hydrolysate (Chart 2) formed by 6 *N* HCl and pH 8.5 hydrolysis of the acidic and amphoteric oligomers (Chart 1) revealed the presence of guanidine. It was detected by positive color tests with the pentacyanoferrate and the diacetyl reagents (Smith, 1969). A yield of 3.3-4.4 mg/l of the oligomerization mixture was established by comparison of the

intensity of the guanidine spot, obtained by acid hydrolysis of 5 mg of the acid and amphoteric oligomers, with varying amounts of authentic guanidine applied to the same paper chromatogram. A guanidine yield of 3.5-4.5 mg/l and 2.5-3 mg/l was obtained by 6 *N* HCl hydrolysis and pH 8.5 hydrolysis, respectively, of oligomers formed in the absence of oxygen.

Guanidinoacetic Acid. Guanidinoacetic acid was detected in the basic and amphoteric hydrolysate (Chart 2) of the acidic and amphoteric oligomers (Chart 1) by the identity of R_f value with an authentic sample when measured in systems 1, 2 and 7. The compound was detected using the pentacyanoferrate and diacetyl reagents (Smith, 1969). The yield of guanidinoacetic acid was found to be 0.8 mg/l of the oligomerization mixture after acid hydrolysis and 0.4 mg/l after pH 8.5 hydrolysis.

Identification of Amino Acids by GC/MS of the (+)-2-Butyl Ester Trifluoroacetamide Derivatives

A. Preparation of (+)-2-Butyl-N-trifluoroacetamides of Amino Acids. To a 1-2 mg sample of the amino acid mixture was added 0.5 ml of 3 N HCl in (+) 2-butanol. The mixture was sonicated for a minute to facilitate dissolution of the sample and then heated in a sealed tube for 2 h at 100-150°C. The solution was concentrated to dryness with warming to 60°C, 1 ml of CH_2Cl_2 was added and the solution was again concentrated to dryness with warming. The residue was taken up in 0.8 ml of CH_2Cl_2 and 0.3 ml of trifluoracetic anhydride was added. The mixture was sonicated for one minute and allowed to stand at room temperature for 1 h. The mixture was concentrated to dryness using a rotary evaporator and a room temperature water bath. CH_2Cl_2 (0.2 ml) was added and the mixture was again concentrated and the residue was dissolved in 0.1 ml of CH_2Cl_2 for gas chromatography.

B. Gas Chromatography/Mass Spectrometry of the (+)-2-Butyl trifluoroacetamide Derivatives of Amino Acids. Samples (2 μl) were chromatographed on a 150 ft x 0.02 in. Ucon 75-H-90,000 capillary column using a model 7620A Hewlett-Packard Gas Chromatograph (Gil-Av et al., 1965; Pollock et al., 1965). The column was held for 8 min at 100° and programmed at 10/min. to 160°. The helium flow rate was 6 ml/min, make up helium was 47 ml/min, air 300 ml/min and hydrogen 30 ml/min. This column gave fair (not baseline) separation of the diastereomeric derivatives of valine, alanine, isoleucine, leucine, proline, phenylalanine and glutamic acid but not aspartic acid.

The acidic and amphoteric oligomers and the basic oligomers (Chart 1) were hydrolyzed and the basic and amphoteric hydrolysate (Chart 2) was analyzed (Table 2). With the exception of the isomers of diaminosuccinic acid the same amino acids were detected for each preparation. The yield of amino acids was much greater with the acid hydrolysis than with pH 8.5 hydrolysis. N-methylglycine and β-alanine were not observed in the previous study which utilized the l-butyl-N-trifluoroacetamide derivatives (Ferris et al., 1974a). No glutamic acid or isoleucine, previously reported compounds, were detected in the present work. Previously the glutamic acid was only observed on hydrolysis of the neutral fraction of the HCN oligomers, a fraction which was not investigated in this study. Isoleucine was observed irregularly and in very small yield in the previous study (Ferris et al., 1974a).

Table 2. GC/MS analysis of D-2-butyl TFA amino acids from HCN oligomers[a]

Amino Acid	*Sample*[b]		
	Acid Hydrolysis Acidic and Amphoteric Oligomers	Acid Hydrolysis Basic Oligomers	pH 8.5 Hydrolysis Deoxygenated Acidic and Amphoteric Oligomers
α-aminoisobutyric	M	M	M
alanine	P	P	–
N-methylglycine	P	P	–
ammonia	T	M	M
glycine	S	S	S
β-alanine	M	M	P
aspartic	M	M	M
diaminosuccinic	M	–	P
diaminosuccinic	M	–	P

[a]Identity established by mass spectral analysis with the exception of the pH 8.5 hydrolysis of the deoxygenated oligomers where only the GC retention time was used. The GC trace in the latter case was similar to the other runs

[b]S = strong, M = medium, T = trace, P = possibly present but not in sufficient amount to establish unequivocally

Results and Discussion

The present study was performed to investigate:

1) Which Biomolecules are Formed from Dilute (0.1M) Cyanide Solutions. Since it is unlikely that cyanide concentrations of 1-11*M*, that had been used in previous studies, were ever present on the primitive earth, we investigated 0.1 *M* cyanide solutions. The choice of 0.1 *M* cyanide is a compromise since it represents an absolute upper limit for primitive earth cyanide ion concentrations and a practical lower limit for oligomer formation in the absence of the concentration of the HCN by eutectic formation (Sanchez et al., 1966).

2) The Effect of Molecular Oxygen on the Formation of Biomolecules from HCN. Oxygen had not been excluded in most of the studies carried out previously because it was reported to have no effect on the oligomerization of cyanide (Völker, 1960). Recently it was demonstrated that diaminomaleonitrile (2) is readily air oxidized (Ferris and Ryan, 1973). This result opened the question of a possible role for molecular oxygen in the formation of biomolecules from HCN since there was little or no oxygen in the atmosphere of the primitive earth (Miller and Orgel, 1974).

3) Variation in the Structures of the Biomolecules Released from the HCN Oligomers when Hydrolyses are Performed at pH 8.5. Previously all the hydrolyses were performed with 6 *N* HCl. Neutral hydrolysis conditions are most reasonable since the pH of the primitive earth oceans was probably pH 8 ± 1 (Miller and Orgel, 1974).

In our experimental approach we allowed 0.1 *M* cyanide to condense to oligomers under plausible primitive earth conditions. The acidic and amphoteric oligomers, isolated by ion exchange techniques (Ferris et al., 1974a) were then subjected to

hydrolysis at 110°C. The pH 8.5 hydrolysis of these oligomers simulates their slow breakdown on the primitive earth with the release of the requisite biomolecules essential for the origins of life. It is necessary to do these hydrolyses at 110° in the laboratory so that they proceed at a reasonable rate. It is assumed that neither the nature of the reaction products nor their relative yields is changed by the use of 110° as compared with the longer term, lower temperature hydrolyses that probably occurred on the primitive earth.

The detection of 4,5-dihydroxypyrimidine (9), orotic acid (8) and 5-hydroxyuracil (10) is the first observation of the formation of pyrimidines using HCN as the only starting material (Table 3). The yield of 4,5-dihydroxypyrimidine is especially impressive since it is similar to that of glycine and greater than adenine. The structure of 4,5-dihydroxypyrimidine was deduced by the low resolution and high resolution GC/MS of the bis(trimethylsilyl) derivative (Folsome et al., 1973). This structure was confirmed by a direct mass spectral comparison with an authentic sample prepared by the acid hydrolysis of 4,5-diaminopyrimidine (McOmie and Turner, 1963).

Orotic acid (8) is only observed when the oligomer hydrolysis is performed at pH 8.5. This is probably because 5-carboxymethylidine hydantoin (7), a precursor to orotic acid, is formed by acid hydrolysis and this in turn rearranges to orotic acid only in neutral or basic solution (Ferris et al., 1974b). Presumably these reactions proceed sequentially at pH 8.5 to yield orotic acid.

The adenine yield we observed from oligomers prepared using 0.1 *M* HCN is one-tenth to one-fifth the optimum yields obtained using 2-11 *M* HCN solutions (Oro and Kimball, 1961). This yield is at least ten times higher than expected if the first step in the adenine synthesis is the slow bimolecular reaction of aminomalononitrile (1) with formamidine to give 4-aminoimidazole-5-carbonitrile (5). The concentration of each of these reactants should be decreased at least ten fold in 0.1 *M* HCN as compared to 1 *M* or 10 *M* HCN solutions and therefore the adenine yield should be about one hundredth to one thousandth that observed by Oro and Kimball (1961). Since these results and other results, which will be cited later, demonstrate that formamidine is not a likely reaction intermediate, the principal pathway for adenine synthesis must involve initial formation of HCN oligomers which release adenine on hydrolysis (Scheme 1).

The extent of HCN oligomer formation is a function of the concentrations of cyanide and diaminomaleonitrile. This was demonstrated by the observation that the rate of oligomer formation increased when 2 is added to freshly prepared cyanide solutions (Sanchez et al., 1967). The steady state concentration of 2 is a complex function of cyanide concentration as evidenced by the observation that its steady state concentration from 0.1 *M* cyanide is greater than that from 5 *M* cyanide after a 5 day reaction time (Sanchez et al., 1967). Since the adenine yield does not decrease by the square of the concentration as the cyanide level is decreased from 11 *M* to 0.1 *M*, a bimolecular reaction between aminomalononitrile and formamidine is probably not involved in the synthesis of adenine from 0.1 *M* cyanide. A pathway via diaminomaleonitrile and HCN oligomers is consistent with these data.

Further support for the proposal that formamidine is not involved in adenine synthesis is our observation that both adenine and 4,5-dihydroxypyrimidine are released from oligomers that were prepared in the absence of added NH_3. The ammonia

concentrations (from the hydrolysis of HCN) must be very low and the corresponding concentration of formamidine must be essentially zero. Consequently it is very unlikely that formamidine is involved in the formation of adenine.

Formamidine does have a central role in the HCN oligomerizations performed under anhydrous conditions (Wakamatsu et al., 1966; Yamada et al., 1968, 1969). In the absence of water the HCN oligomerization proceeds at a slower rate and there is no loss of formamidine by hydrolysis. Hence, the higher concentration of formamidine and the slower oligomerization result in the more efficient conversion of aminomalononitrile to adenine by the route shown in Scheme 1 when anhydrous conditions are used.

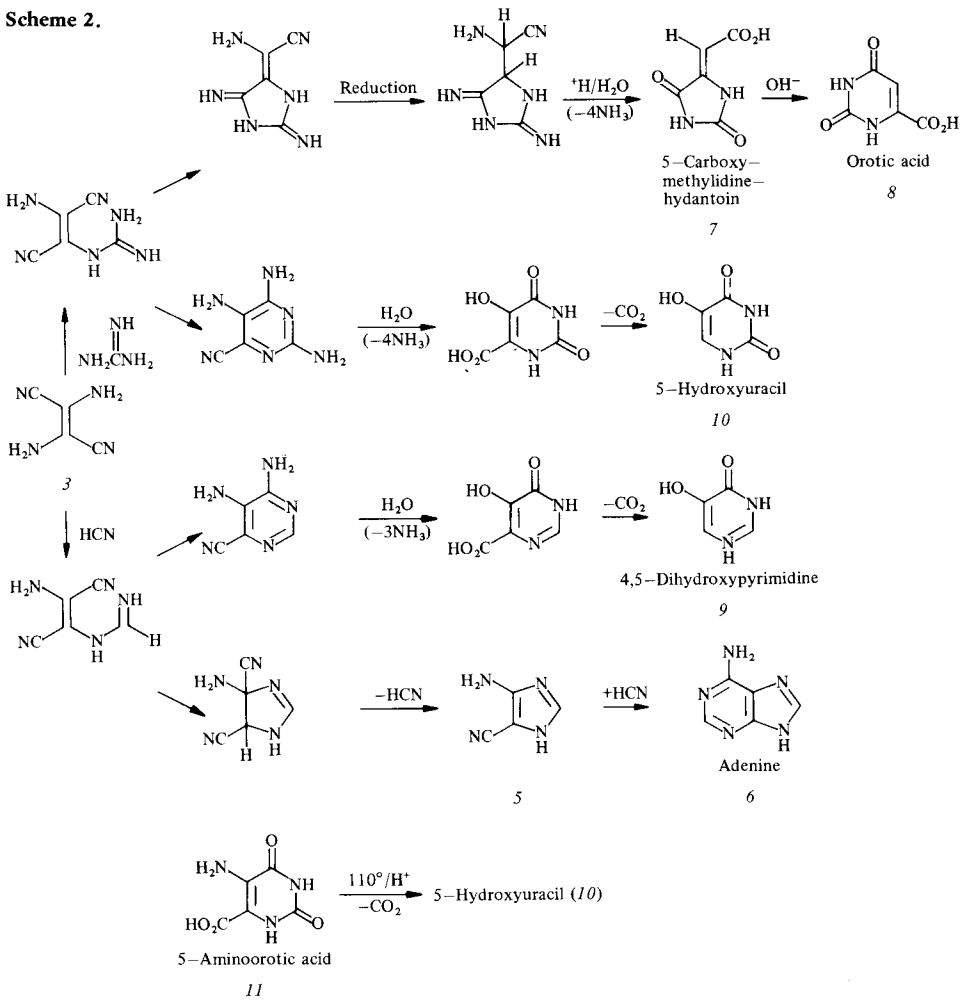
No adenine was detected when the oligomeric mixture from 0.1 *M* cyanide was analyzed before hydrolysis. The absence of free adenine is also consistent with a synthetic pathway via HCN oligomers which release adenine upon hydrolysis. This is also the predominant reaction pathway with concentrated solutions of cyanide as evidenced by the observation that only 16% of the adenine formed from 11.1 *M* cyanide is free in solution (Oro and Kimball, 1961).

The pH 8.5 hydrolyses reported in Table 3 were performed on oligomers which were prepared and hydrolyzed in the absence of oxygen. The same reaction products were obtained when a small amount of molecular oxygen was present during the oligomerization and hydrolysis reactions. Although it has been shown that diaminomaleonitrile (2) is readily oxidized by oxygen (Ferris and Ryan, 1973), these data show that the presence of molecular oxygen does not result in any significant changes in the reaction products. This is probably because all the non-degassed reaction solutions were stoppered and were not agitated during the olgomerization reaction. As a consequence, the oxygen which is initially in solution is scavanged by the initially formed diaminomaleonitrile. Since very little additional oxygen diffuses into the stoppered flasks a steady state concentration of 2 is built up and the oligomerization proceeds. There is a lower steady state concentration of 2 and correspondingly smaller yields of HCN oligomers if the solutions are saturated with oxygen.

A unified pathway for the formation of the heterocyclic compounds listed in Table 3 is given in scheme 2. This Scheme is an oversimplification since these compounds are not found free in solution but are incorporated into HCN oligomers. The final products (6, 8, 9, 10) are obtained by hydrolysis of the oligomers. A pathway similar to that proposed for the formation of 4,5-hydroxypryrimidine (9) and 5-hydroxyuracil has been proposed for the synthesis of 2-substituted-4,5-diamino-6-cyanopyrimidines from diaminomaleonitrile (2) (Begland, 1975). As discussed previously, only HCN is used as a reactant (Scheme 2) with the exception of the proposed role of guanidine or its equivalant in the formation of 5-hydroxyuracil (10). Guanidine is a plausible reactant since it is formed in appreciable yield from HCN (Table 3). The decarboxylation reactions postulated in the formation of 4,5-dihydroxypyrimidine (9) and 5-hydroxyuracil (10) are analogous to the decarboxylation of β-ketoacids (Ferris and Miller, 1966). The validity of the proposed decarboxylation step was shown by our conversion of 5-aminoorotic acid (11) to 5-hydroxyuracil (10) in 29% yield when heated at 110° for 18 h in 6 *N* HCl.

The previous report of the release of amino acids on acidic and pH 8 hydrolysis of the HCN oligomers (Ferris et al., 1974a) was confirmed in this study. The presence

Scheme 2.



of glycine, *meso*- and *dl*-diaminosuccinic acid, α-aminoisobutyric acid and β-alanine was established by GC/MS analysis of their silylated derivatives (Butts, 1972). Alanine, N-methylglycine and aspartic acid were also identified along with glycine, β-alanine, α-aminoisobutyric acid and the diaminosuccinic acid isomers by the GC/MS analysis of the (+)-2-Butyltrifluoracetyl derivatives (Table 2) (Gil-Av et al., 1965; Pollock et al., 1965). The quantitative yields of glycine and diaminosuccinic acid isomers given in Table 3 were obtained using an amino acid analyzer.

Table 3. Biomolecules from hydrolysis of HCN oligomers[a]

	6 *N* HCl	pH 8.5[b]
4,5-dihydroxypyrimidine	0.7 –0.9%	–
orotic acid (*8*)	–	0.009%
5-hydroxyuracil (*10*)	0.003%	–[d]
pyrimidine (structure unknown)[b]	–	+
adenine (*6*)	0.03–0.04%	+
4-aminoimidazole-5-carboxamide (*4*)	+	+[e]
glycine	0.6%	0.1%
diaminosuccinic acid	0.1%[c]	+
aspartic acid	+	+
alanine	+	+
β-alanine	+	+
α-aminoisobutyric acid	+	+
guanidinoacetic acid	0.03%	+
guanidine	0.2%	0.1%

[a]HCN oligomers were prepared and hydrolyzed as described in the Experimental section. The yields shown are based on starting HCN. Conversions are approximately 2x as great since only about 50% of the cyanide is consumed. It is assumed that 5 moles of HCN are required to form each mole of heterocyclic compound and 4 moles are required to form each mole of glycine. The identity of each compound was established by comparison of the mass spectrum of a volatile derivative with that of an authentic sample except for the following which were purified by chromatography and identified by their UV spectra and/or specific color tests: adenine, 4-aminoimidazole-5-carboxamide, guanidinoacetic acid and guanidine. The presence of the compound is indicated by (+) and absence by (–). When quantitative analyses were not performed no analysis is indicated by (nd). The presence of guanine, xanthine and hypoxanthine is suggested by UV data but these were not reported because their identity has not been confirmed by mass spectral data.

[†]Products from oligomers prepared from degassed HCN solutions. Hydrolyses were performed in evacuated ampuls.

[b]This compound gives a blue color with diazotized sulfanilic acid and it is converted to 4,5-dihydroxypyrimidine by hydrolysis with 6 *N* HCl.

[c]Sum of the yields of *meso*- and *dl*-diaminosuccinic acid. They are formed in approximately a 2:1 ratio respectively.

[d]Control experiments demonstrated that this compound is destroyed by the hydrolysis conditions used in this study.

[e]4-aminoimidazole-5-carboxamide decomposes under acid hydrolysis conditions and when hydrolyzed at pH 8.5 for 24 h. It can be detected in the hydrolysate of the HCN oligomers formed in the absence of oxygen when a 1.5 h hydrolysis time is used.

HCN and Chemical Evolution

Representatives of the three major classes of nitrogen containing biomolecules, purines, pyrimidines and amino acids are formed in comparable amounts by the hydrolysis of the oligomers formed from dilute aqueous solutions of HCN. Since these biomolecules are released from HCN oligomers when either acidic or mildly basic hydrolysis conditions are used it is likely that they would have been formed from the HCN oligomers

even if there were large differences in the pH in different locales on the primitive earth. HCN is produced under a variety of plausible primitive earth conditions so it seems very likely that it condensed to these oligomers on the primitive earth. Although these biomolecules can be formed from other reactants (Miller and Orgel, 1974; Ferris et al., 1974c; Chittenden et al., 1976; Miller, 1970) it is particularly impressive that all three structural types are also formed directly from HCN. This primitive earth synthesis is more compelling than those which require different reactants and reaction conditions for each structural type. Although we do not maintain that HCN was the sole source of biomolecules, it is not nescessary, starting from HCN, to postulate a variety of environments on the primitive earth in order to account for the formation of each different structural type of biological molecule.

Two of the pyrimidines obtained from HCN oligomers are present in contemporary biological systems. Orotic acid is a central intermediate in the biosynthesis of pyrimidine nucleotides (Lieberman et al., 1955). 5-Hydroxyuracil is a minor component of yeast RNA (Lis and Passarge, 1966; Hayes and Lis, 1973) and its derivative, uracil-5-oxyacetic acid is present in the anticodons of several transfer RNA's (Yanio and Barrell, 1969; Murao et al., 1970; Weiss, 1973). The biochemical similarity of uridine and 5-hydroxyuridine was demonstrated by the observation that 5-hydroxyuridine undergoes most of the metabolic transformations of uridine in Ehrlich ascites cells (Smith and Vissar, 1965) and is hydrolyzed with yeast nucleoside hydrolase (Lis and McLaughlin, 1971). These findings suggest that 5-hydroxyuracil is compatible with contemporary biochemical processes and consequently it may have been utilized in primitive nucleic acids.

Although the attempted prebiotic syntheses of pyrimidine nucleosides starting from cytosine, uracil or thymine has not been successful (Fuller et al., 1972a,b) we feel that such a synthesis is likely if 4,5-dihydroxypyrimidine or 5-hydroxyuracil is used in their place. The previous lack of success probably reflects the limited nucleophilicity of the non-basic heterocyclic nitrogen atoms present in these pyrimidines. These would be poor nucleophiles and would not effectively displace groups present in the 1'-position or ribose. The successful synthesis of purine nucleosides under the same reaction conditions can be explained by the presence of the nucleophilic imidazole nucleus (Fuller et al., 1972a,b). This prebiotic purine synthesis is very similar to the "salvage" pathway for the biosynthesis of purine nucleotides in which the purine base displaces the pyrophosphate grouping from the 1-position of 5-phosphorylribose-1-pyro–pyrophosphate (Lehninger, 1975). It seems likely that 4,5-dihydroxypyrimidine, which contains a basic heterocyclic nitrogen, would react with ribose derivatives to give the corresponding nucleoside. A similar argument can be made for 5-hydoxyuracil and the metal chelates of orotic acid.

Intermediates in the contemporary biosynthesis of purines and pyrimidines are also formed from dilute cyanide solutions. Orotic acid (8) and 4-aminoimidazole-5-carboxamide (4), which as the ribotides serve as precursors to pyrimidines and purines respectively, are both present. Their formation from HCN and their central role in contemporary biosynthetic pathways suggests that when the supply of preformed purines and pyrimidines became limiting, only those primitive life forms which could synthesize purines and pyrimidines survived (Horowitz, 1945). A provocative corollary to this hypothesis is that some of the fundamental metabolic pathways in contemporary

life may have been established several billion years ago as a consequence of the reaction products formed from dilute aqueous solutions of HCN.

The present study demonstrated that formamidine is not essential for the formation of purines in the absence of UV light. Previously it was believed that formamidine was an essential intermediate in the synthesis of adenine (Sanchez et al., 1967). It was felt that this constituted a serious constraint of adenine synthesis from HCN because formamidine is rapidly hydrolyzed under primitive earth conditions. As a consequence a route to adenine was proposed which required the photochemical rearrangement of 2 (Scheme 1) (Sanchez et al., 1967; Ferris and Orgel, 1966a,b; Ferris et al., 1969; Ferris and Kuder, 1970). We now consider the photochemical pathway and the non-photochemical pathway via HCN oligomers to be plausible complementary routes for purine synthesis on the primitive earth.

A primitive earth scenario for the synthesis of these biomolecules would be the constant formation of HCN by the action of electric discharges (Sanchez et al., 1967) shock waves (Bar-Nun and Tauber, 1972) and ultraviolet light (Hubbard et al., 1975; Ferris and Chen, 1975) on the primitive atmosphere. The HCN would be dissolved in rain droplets and carried to the earth's surface where it would oligomerize in lakes and oceans (Sanchez et al., 1967; Miller and Orgel, 1974). These oligomers slowly hydrolyze to yield the biomolecules which eventually organized into the primitive forms of life.

Appreciable amounts of biomolecules could have formed on the primitive earth from the HCN dissolved in lakes and rivers. A one liter solution of 0.1 *M* cyanide yields, in one year, sufficient HCN oligomers to give 7.5 μmol adenine, 130 μmol 4,5-dihydroxypyrimidine and 160 μmol glycine after hydrolysis. A small primitive lake the size of Lake George, New York (volume 2 km^3 or 2 x 10^{12} liters) could yield sufficient HCN oligomers annually to give about 10^7 mol of adenine and 10^8 mol of 4,5-dihydroxypyrimidine and glycine if it contained 0.1 *M* cyanide. A lake the size of Lake Superior would have the potential of producing 6000 times as much of these biomolecules each year. The quantities of these compounds would increase more than a 1000 fold over 10^3–10^6 years. While it is recognized that there are many assumptions lurking in these simple calculations (e.g. inefficient synthesis of cyanide or its complexation with metal ions or hydrolysis of the cyanide) which could change the final values by several orders of magnitude, the results demonstrate that significant quantities of all three classes of nitrogen-containing biomolecules may have been formed exclusively from HCN on the primitive earth.

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