Origins of Life and Evolution of Biospheres (2005) 35: 79–90

AN INVESTIGATION OF PREBIOTIC PURINE SYNTHESIS FROM THE HYDROLYSIS OF HCN POLYMERS

EDUARDO BORQUEZ[1,3], H. JAMES CLEAVES[1], ANTONIO LAZCANO[2*] and STANLEY L. MILLER[1]

[1] *Department of Chemistry and Biochemistry, University of California, San Diego, La Jolla, CA, U.S.A.;* [2] *Facultad de Ciencias, UNAM, Cd. Universitaria, Mexico, D.F., Mexico;* [3] *Current address: 370 Longwood Ave. #47, Boston, MA, U.S.A.*

(author for correspondence, e-mail: alar@correo.unam.mx)*

(Received 8 April 2003; accepted in revised form 4 November 2003)

Abstract. The polymerization of concentrated NH_4CN solutions has been studied at various temperatures and ammonia concentrations. The products of the oligomerization of ammonium cyanide include adenine and guanine, as well as trace amounts of 2,6-diaminopurine. Our results indicate that the adenine yield is not strongly dependent on temperature. Guanine is produced in lower yield. The original studies by Oró and Kimball (1961) showed that the 6 N HCl hydrolysis of the NH_4CN polymerization supernatant greatly increased the adenine yield. However, this hydrolysis also decomposes adenine and other purines. Therefore, we have measured the yields from an NH_4CN polymerization as a function of hydrolysis time, and found that shorter hydrolytic periods give higher yields of adenine. We have also investigated the hydrolysis of the supernatant at pH 8, which is a more reasonable model of primitive oceanic conditions, and found that the adenine yield is comparable to that obtained with acid hydrolysis (approximately 0.1%). The yield of adenine does not decline at longer hydrolysis times because of the greater stability of adenine at pH 8. The insoluble black polymer formed from NH_4CN has been analyzed by both acid and neutral hydrolysis. In both cases adenine yields of approximately 0.05% were obtained. This suggests that the polymer may have been as important a prebiotic source of purines as the usually analyzed supernatant.

Keywords: 2,6-diaminopurine, adenine, guanine, NH_4CN polymerization

1. Introduction

The synthesis of adenine from the polymerization of concentrated ammonium cyanide solutions at high temperatures (80 to 90 °C) is well documented (Oró, 1960; Oró and Kimball, 1961, 1962). Since it was first reported, the prebiotic synthesis of adenine from the polymerization of HCN has been investigated in considerable detail under various conditions. Variations of adenine synthesis have been carried out using a photochemical rearrengement step (Sanchez *et al*., 1967), with formaldehyde and other aldehydes as catalysts (Schwartz and Goverde, 1982; Voet and Schwartz, 1983), from heating formamide at high temperatures (160 °C) in the presence of various metallic oxides (Saladino *et al*., 2001) and, more recently, from the reaction of the HCN tetramer with ammonium formate (Hill and Orgel, 2002). Guanine and other purines have been reported by a three-step reaction scheme with

frozen HCN solutions that react to form the HCN tetramer (diaminomaleonitrile) (Sanchez *et al*., 1966) followed by a two-photon rearrangement to amino imidazole carbonitrile (AICN) (Ferris and Orgel, 1966), which then reacts with cyanogen or cyanogen bromide (Sanchez *et al*., 1967, 1968). More recently, guanine has been obtained, together with adenine and 2,6-diaminopurine, as a product of the polymerization of NH_4CN in a 'one pot synthesis' (Levy *et al*., 1999).

Oró and Kimball (1961) also showed that the 6 N HCl hydrolysis at 100 °C of the NH_4CN polymerization mixture supernatant greatly increased the adenine yield. However, the hydrolysis conditions originally used by Oró and Kimball (1961, 1962) also lead to significant decomposition of adenine and other purines (Levy and Miller, 1998). Here we report the presence of adenine, guanine and trace amounts of 2,6-diaminopurine as products of the NH_4CN polymerization mixture after neutral hydrolysis. We have measured the adenine yield at various concentrations of ammonium cyanide and ammonia, and at reaction temperatures between –78 to 100 °C. In order to study whether the high temperature/high concentration reaction would proceed under other conditions, these results were compared with a long-term experiment performed in this laboratory in which solutions of NH_4CN were kept frozen continuously at –78 and –20 °C for 25 yr (Levy *et al*., 2000). We also report here the results of the study of the hydrolysis at pH 8 of the supernatant, which may be a more realistic prebiotic model. Finally, the products of both acid and neutral hydrolysis of the black polymer were studied, in order to explore the possibility of this additional prebiotic source of purines.

2. Material and Methods

Ammonium chloride and sodium cyanide were obtained from Fisher Scientific. Adenine, guanine and 2,6-diamino purine standards were obtained from Calbiochem. Ammonium cyanide was prepared by mixing NH_4Cl with NaCN. The solution quickly precipitated a black polymer on heating. All the reactions, including the hydrolytic ones, were performed in sealed 5 mL glass ampoules, except for the dilute (<1 M NH_4CN) polymerizations, which were carried out in round bottom flasks degassed before reaction and hydrolyzed under reflux. Reaction mixtures were kept at constant temperature on Thermolyne 16500 dry heating blocks (ampoules) or in thermostatted oil baths (round bottom flasks). All samples were centrifuged after reaction, and the supernatant separated from the insoluble black polymer. Acid hydrolysis of the supernatant was conducted by diluting the reaction 1:1 with 12 N HCl. The neutral hydrolysis was conducted by diluting the supernatant 1:10 in 0.1 M pH 8 phosphate buffer. The polymer was washed once with water, dried, and weighed. The acid hydrolysis of the polymer was conducted by diluting the polymer to 0.03 g mL^{-1} in 6 N HCl, and the neutral hydrolysis by diluting the polymer to 0.012 g mL^{-1} in pH 8, 0.1 M sodium phosphate buffer. For analysis, samples were suspended in 0.01 N HCl to increase solubility (De-

voe and Wasik, 1984), and filtered through 0.22 μ syringe filters. All samples were first fractionated using DOWEX AG50Wx8 columns (H+ form), using HCl as eluant as described in Levy *et al.*, (2000). For HPLC analysis, the adenine containing fractions from the ion exchange columns were suspended in 0.01 N HCl to increase solubility (Devoe and Wasik, 1984), and filtered through 0.22 μ syringe filters. HPLC analysis was conducted using a Waters YMC ODS-AQ analytical reversed phase column (260×6 mm) eluted with 0.1 M pH 4.8 potassium phosphate buffer/5% (v/v) methanol using two Beckman 110B pumps. The eluant was monitored using a Kratos 757 UV absorbance detector set at 260 nm. Peaks corresponding to adenine, guanine and 2,6-diaminopurine were collected and re-chromatographed using 0.1 M pH 2.5 potassium phosphate buffer. Products were identified by retention times and UV absorption spectra using a Hewlett Packard 8452A Diode Array Spectrophotometer using 1 cm path-length quartz cuvettes. Purine yields, unless otherwise stated, were calculated based on carbon input in the form of sodium cyanide as mole percentages using the maximum theoretical yield (5 moles NaCN per mole of adenine). The mechanisms of formation of guanine and 2,6-diaminopurine presumably involve other species generated in the reaction (e.g. cyanate and cyanogen, respectively). Since the concentrations of these species are difficult to determine during the course of the reaction, the yields of these two purines were calculated using the same method used for adenine (i.e., 5 moles NaCN per mole purine). Experiments were conducted three times for each data set.

3. Results

Figure 1 shows an HPLC chromatogram of purine-containing fractions from DOWEX-50 chromatographic separation of the hydrolyzed insoluble polymer obtained from an NH_4CN polymerization at 80 °C. The peaks assigned to adenine, guanine and 2,6-diaminopurine were identified by chromatographic retention time, co-injection with standards, and correlation of the UV spectra of the peaks with standard spectra. Acid hydrolysis of the polymer gave a yield of adenine of 0.04% (weight percent based on polymer dry weight) as opposed to the 0.13% (mole percent based on starting NaCN) of the corresponding supernatant. It is difficult to determine the mole percent yields of adenine from the polymer since its composition is unknown, thus a direct comparison of these percentages is unwarranted. Guanine was produced in yields 10 to 40 times less than adenine.

In order to study the effect of temperature on the NH_4CN polymerization reaction, the adenine yields obtained from the high-temperature experiments reported here were compared with yields from a long term, low-temperature experiment in which samples of 0.1 M NH_4CN that were left frozen at –78 and –20 °C for 25 yr (Levy *et al.*, 2000), then thawed and analyzed as for the 80 °C samples. As shown in Figure 2, the data suggest that the adenine yield is not strongly dependent on

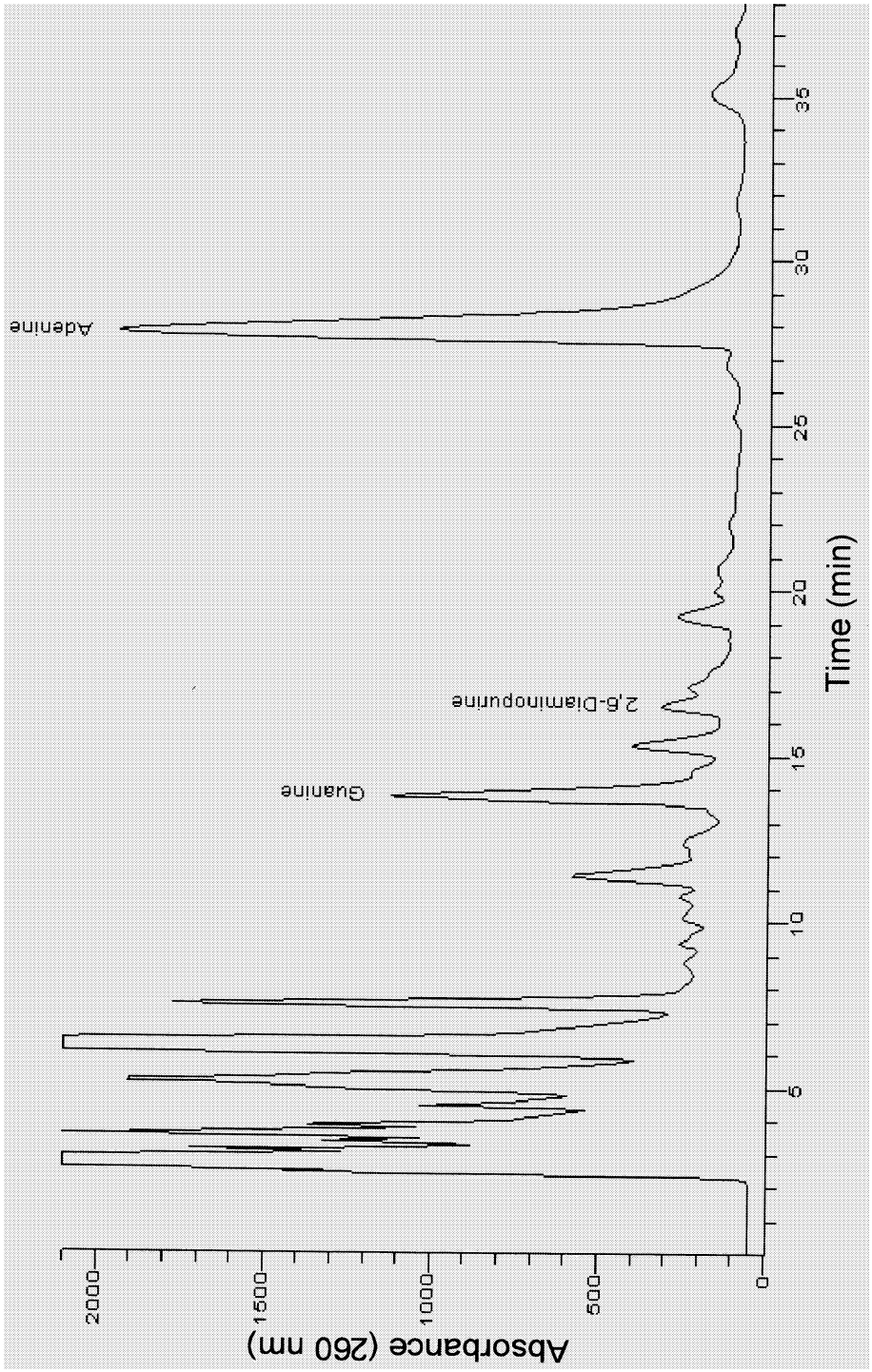

Figure 1. HPLC chromatogram (Using a Waters YMC ODS-AQ (260×6 mm) analytical reversed phase column) of the polymer (YMC ODS-AQ 4.6×250 mm column) from a 10 M NH4CN polymerization conducted at 80 °C for 13 days, after 6 N HCl hydrolysis at 100 °C for 24 hr.

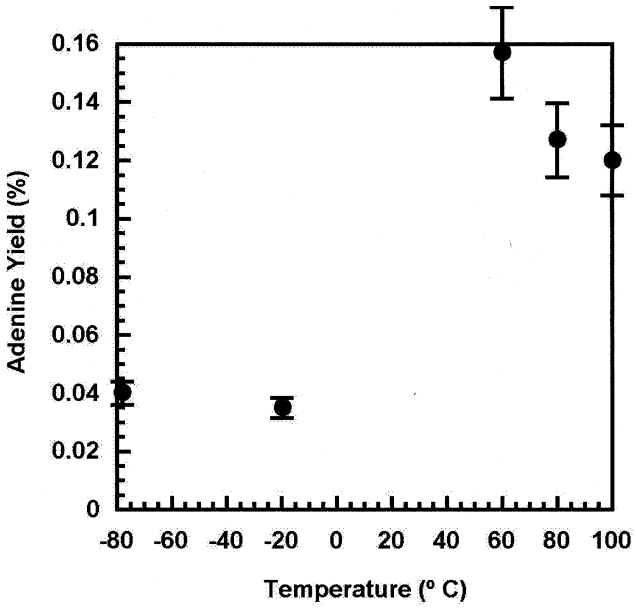

Figure 2. Yield of adenine from the unhydrolyzed supernatant as a function of polymerization temperature from 0.1 M NH_4CN at infinite time.

temperature. This suggests that the high-temperature experiments are a reasonable model of the reactions that take place at low temperature on a geological time scale.

The results of the polymerizations at varying concentrations of NH_4CN are shown in Figure 3. The results indicate that the percent yield at high concentrations is approximately proportional to the square of the NH_4CN concentration rather than the first order dependence expected if the rate-limiting step in the polymerization reaction is the attack on HCN by CN^- to form a dimer. The predicted (Bada *et al.*, 1994) sharp drop in the percentage of adenine yield at concentrations <1 M is clearly noticeable. The linear dependence of adenine yield on ammonia concentration was also investigated and the results are summarized in Figure 4.

Initial experiments by Oró and Kimball (1961) indicated that acid hydrolysis of the supernatant substantially increased the adenine yield. However, these conditions also hydrolyze adenine and guanine (Levy and Miller, 1998). The percent yield of adenine as a function of 6 N HCl hydrolysis time shown in Figure 5 demonstrate that decomposition of adenine induced by this procedure can lead to lower yields. To overcome this problem, we have investigated the hydrolysis of the NH_4CN supernatant under conditions where adenine is more stable and which may be considered more prebiotically relevant. The percent yield of adenine as a function of neutral hydrolysis time at 120 and 140 °C is shown in Figure 6. The total yield is comparable to that obtained with acid hydrolysis (approximately 0.1%), but with no drop in adenine concentration over time. In these experiments

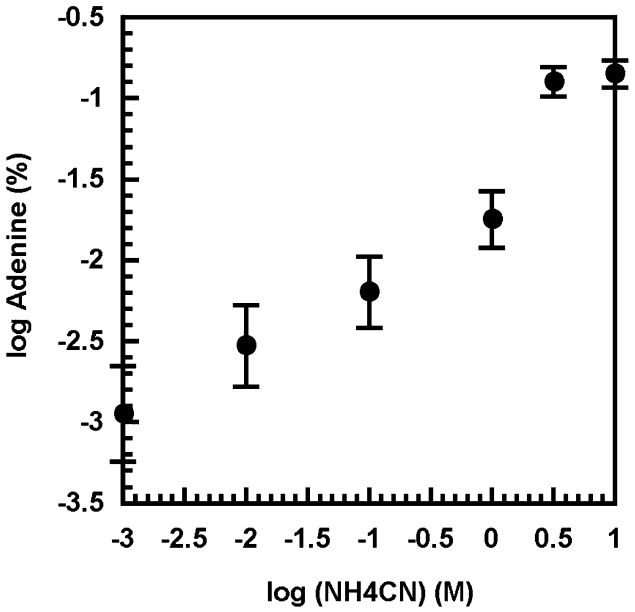

Figure 3. Adenine yield from the unhydrolyzed supernatant as a function of NH_4CN concentration at 80 °C after 16 days.

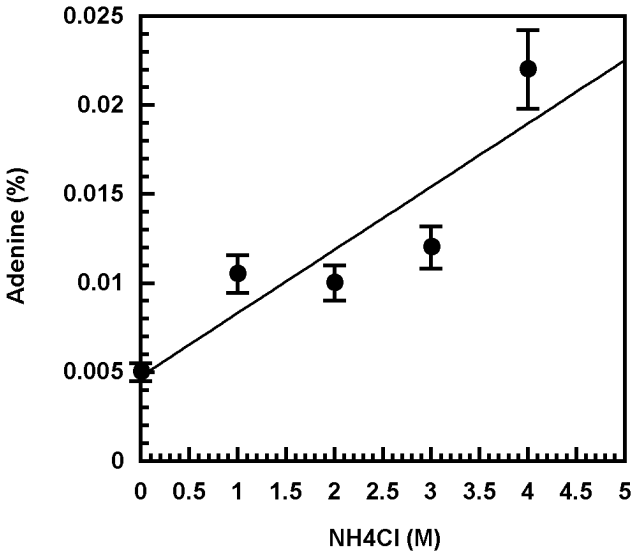

Figure 4. Yield of adenine from the unhydrolyzed supernatant of 6 m HCN as a function of ammonia concentration at 80 °C after 11 days.

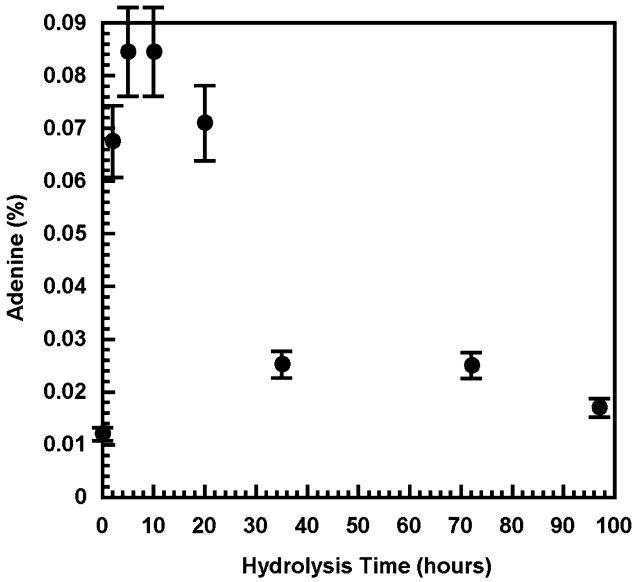

Figure 5. Adenine yield from the supernatant from an 80 °C 10 M NH_4CN polymerization conducted for 48 hr as a function of hydrolysis time in 6 N HCl at 100 °C.

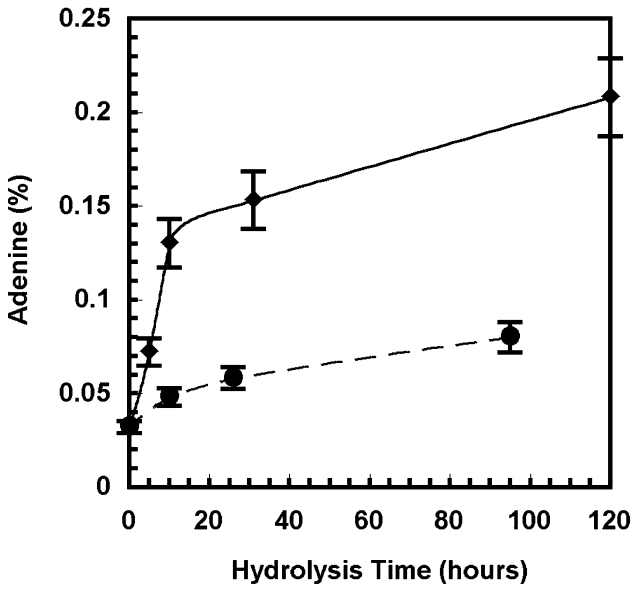

Figure 6. Adenine yield from the supernatant from an 80 °C 10 M NH_4CN polymerization conducted for 48 hr as a function of hydrolysis time in 0.1 M pH 8 phosphate at 120° (circles) or 140 °C (diamonds).

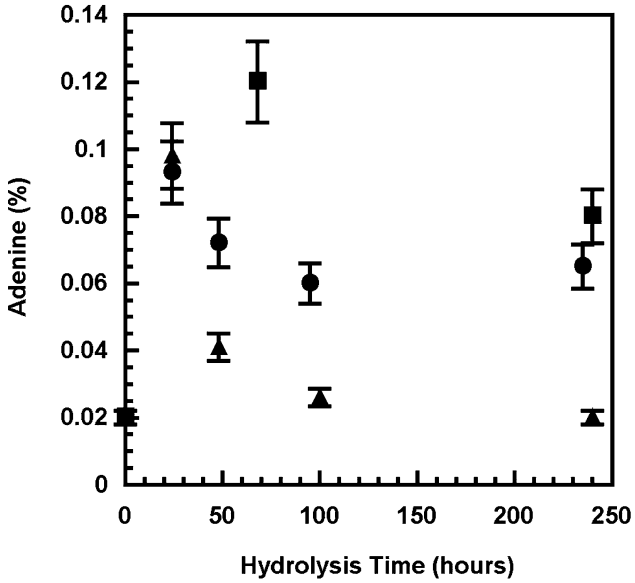

Figure 7. Adenine yield from the insoluble polymer from an 80 °C 10 M NH_4CN polymerization conducted for 48 hr as a function of hydrolysis time in 6 N HCl at 60 (squares), 80 (circles) or 100 °C (triangles).

the yields did not change appreciably if the hydrolysis conditions were acidic or neutral.

We have performed analogous reactions at various temperatures with the insoluble black polymer obtained in NH_4CN polymerizations. As shown in Figure 7, the adenine yields of the acid hydrolysis of the polymer (0.1% adenine yield based on weight adenine/polymer) are comparable to those observed when the supernatant was hydrolyzed under acidic conditions. The acid hydrolysis of the polymer and the supernatant both lead to the characteristic rapid increase and then decrease of adenine production over time at different temperatures. The hydrolysis of the black polymer was also studied under neutral conditions. As shown in Figure 8, this leads to a continuous release of adenine (approximately 0.05%), which is observed at various temperatures. The AG ratios observed at acidic and neutral hydrolysis of the supernatant as shown in Figures 9 and 10, respectively.

4. Discussion and Conclusions

Numerous experiments suggest that HCN could have served as a prebiotic precursor of purines, pyrimidines, and amino acids, as well as of other compounds such as oxalic acid and guanidine (Ferris *et al.*, 1973, 1978; Voet and Schwartz, 1983). Although the formation of adenine via the oligomerization of hydrogen

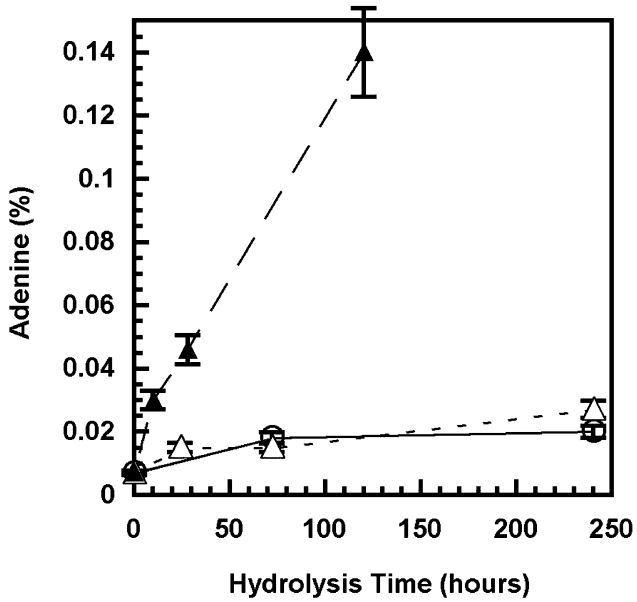

Figure 8. Adenine yield from the insoluble polymer from an 80 °C 10 M NH_4CN polymerization conducted for 48 hr as a function of hydrolysis time in 0.1 M pH 8 phosphate at 100 (open circles), 120 (open triangles) or 140 °C (filled triangles). The effect of buffer catalysis was not investigated.

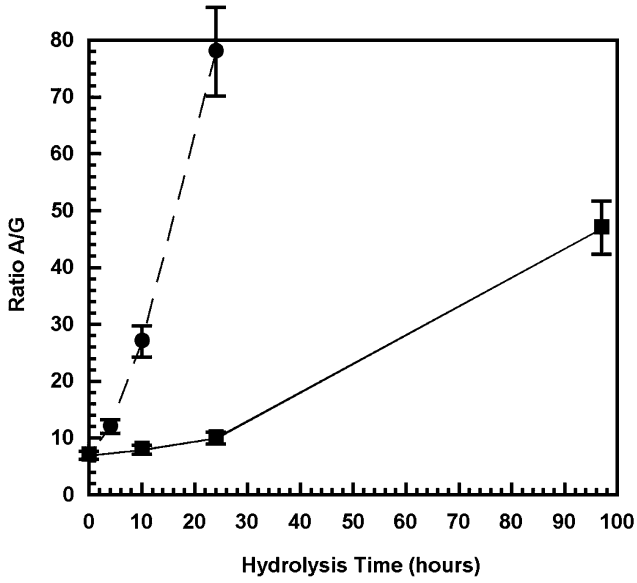

Figure 9. Ratio of adenine to guanine produced from the 6 N HCl hydrolysis of the supernatant of a 10 M NH_4CN polymerization conducted at 80 °C for 48 hr.

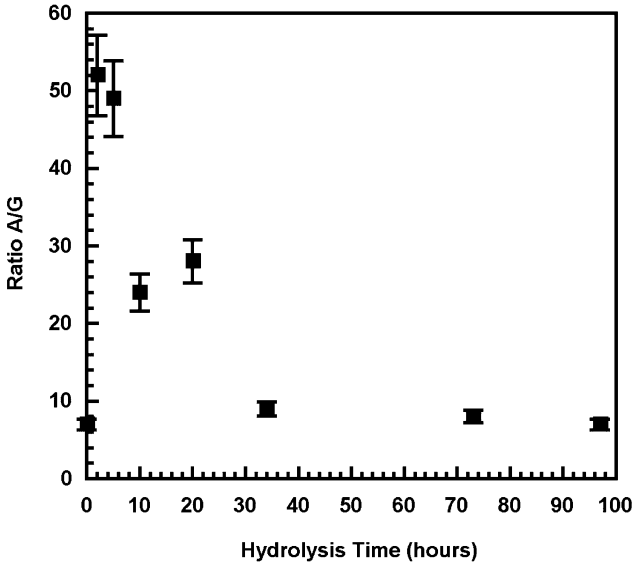

Figure 10. Ratio of adenine to guanine produced from the pH 8 hydrolysis of the supernatant of a 10 M NH_4CN polymerization conducted at 80 °C for 48 hr.

cyanide is well-established, the presence of 2 and 8-cyano adenine in oligomerizing HCN solutions suggests that the actual mechanism may be quite complex, involving the synthesis and rearrangement of at least hexamers and heptamers of HCN (Schwartz, 1998). A possible general mechanism for the synthesis of adenine, guanine and 2,6-diaminopurine involving NH_3, cyanogen and formamidine, which is known to be derived from HCN and NH_3 (Ferris and Orgel, 1966), has been discussed elsewhere (Levy *et al*., 1999).

The presence of adenine, guanine, and 2,6-diaminopurine reported here is consistent with previous results (Ferris *et al*., 1978). The low yields of 2,6-diaminopurine we have observed may be due both to a less efficient synthesis of this base, and to its hydrolytic decomposition into guanine, isoguanine, and xanthine.

We have shown here that adenine is released from the insoluble NH_4CN polymer after acidic or neutral hydrolysis. This suggests that purines could have been released under a wide variety of environmental conditions from any NH_4CN polymer formed on the primitive Earth. The substantial yield of adenine from the hydrolysis of the insoluble polymer suggests that it is an important prebiotic source of adenine. The instability of adenine to hydrolysis previously has been cited as a factor that would limit its prebiotic availability (Shapiro, 1995). This conclusion, however, is based on the rate of hydrolysis of adenine under acid conditions (Frick *et al*., 1987) where the $\Delta H\ddagger$of hydrolysis is much smaller than at neutral pH. Indeed, the neutral hydrolysis of the polymer reported here suggests that purines are released over time, constituting a possible time-release mechanism for adenine and other purines which circumvents some of the stability problems discussed else-

where (Levy and Miller, 1998). Because the rates of synthesis and the hydrolytic stability of both adenine and guanine are similar (Levy and Miller, 1998), it is reasonable to assume that both purines may have been present in the prebiotic environment.

Acknowledgements

We thank the NASA Specialized Center of Research and Training for NSCORT fellowships (H.J.C., A.L.) and grant support (S.L.M.), and an NSCORT summer research fellowship (E.B.).

References

Bada, J. L., Bigham, C. and Miller, S. L.: 1994, Impact Melting of Frozen Oceans on the Early Earth: Implications for the Origin of Life', *Proc. Natl. Acad. Sci. USA* **91**, 1248–1250.

Devoe, H. and Wasik, S. P.: 1984, Aqueous Solubilities and Enthalpies of Solution of Adenine and Guanine, *J. Sol. Chem.* **13**, 51–60.

Ferris, J. P. and Orgel, L. E.: 1966, An Unusual Photochemical Rearrangement in the Synthesis of Adenine from Hydrogen Cyanide, *J. Am. Chem. Soc.* **88**, 1074.

Ferris, J. P., Donner, D. B. and Lobo, A. P.: 1973, Possible Role of Hydrogen Cyanide in Chemical Evolution. Investigation on the Proposed Direct Synthesis of Peptides from Hydrogen Cyanide, *J. Mol. Biol.* **74**, 499–508.

Ferris, J. P., Joshi, P. C., Edelson, E. H. and Lawless, J. G.: 1978, HCN: A Plausible Source of Purines, Pyrimidines, and Amino Acids on the Primitive Earth, *J. Mol. Evol.* **11**, 293–311.

Frick, L., MacNeela, J. P. and Wolfenden, R.: 1987, Transition State Stabilization by Deaminases: Rates of Nonezymatic Hydrolysis of Adenosine and Cytidine, *Bioorganic Chem.* **15**, 100–108.

Hill, A. and Orgel, L. E.: 2002, Synthesis of Adenine from HCN Tetramer and Ammonium Formate, *Orig. Life Evol. Biosph.* **32**, 99–102.

Levy, M. and Miller, S. L.: 1998, The Stability of RNA Bases: Implications for the Origin of Life, *Proc. Natl. Acad. Sci. USA* **95**, 7933–7938.

Levy, M., Miller, S. L. and Oró, J.: 1999, Production of Guanine from NH_4CN Polymerizations, *J. Mol. Evol.* **49**, 165–168.

Levy, M., Miller, S. L., Brinton, K., Bada, J. L.: 2000, Prebiotic Synthesis of Adenine and Amino Acids under Europa-like Conditions, *Icarus* **145**, 609–613.

Oró, J.: 1960, Synthesis of Adenine from Ammonium Cyanide, *Biochem. Biophys. Res. Comm.* **2**, 407–412.

Oró, J. and Kimball, A. P.: 1961, Synthesis of Purines under Primitive Earth Conditions. I. Adenine from Hydrogen Cyanide, *Arch. Biochem. Biophys.* **94**, 217–227.

Oró, J. and Kimball, A. P. 1962, Synthesis of Purines under Primitive Earth Conditions. II. Purine Intermediates from Hydrogen Cyanide, *Arch. Biochem. Biophys.* **96**, 293–313.

Saladino, R., Crestini, C., Costanzo, G., Negri, R. and Di Mauro, E.: 2001, A Possible Prebiotic Synthesis of Purines: Adenine, Cytosine and 4(3H)-pyrimidinone from Formamide: Implications for the Origin of Life, *Bioorganic Med. Chem.* **9**, 1249–1253.

Sanchez, R. A., Ferris, J. P. and Orgel, L. E.: 1966 Conditions for Purine Synthesis: Did Prebiotic Synthesis Occur at Low Temperatures? *Science* **153**, 72–73.

Sanchez, R. A., Ferris, J. P. and Orgel, L. E.: 1967, Studies in Prebiotic Synthesis. II. Synthesis of Purine Precursors and Amino Acids from Aqueous Hydrogen Cyanide, *J. Mol. Biol.* **30**, 223–253.

Sanchez, R. A., Ferris, J. P. and Orgel, L. E.: 1968, Studies in Prebiotic Synthesis. IV. The Convertion of 4-aminoimidazole-5-carbonitrile Derivatives to Purines, *J. Mol. Biol.* **38**, 121–128.

Schwartz, A. W.: 1998, 'Origins of the RNA World', in A. Brack (ed.), *The Molecular Origins of Life: Assembling Pieces of the Puzzle*, Cambridge University Press, New York, pp. 237–254.

Schwartz, A. W. and Goverde, M.: 1982, Acceleration of HCN Oligomerization by Formaldehyde and Related Compounds: Implications for Prebiotic Synthesis, *J. Mol. Evol.* **18**, 351–353.

Schwartz, A. W., Voet, A. B. and Van Der Veen, M.: 1984, Recent Progress in the Prebiotic Chemistry of HCN, *Orig. Life* **14**, 91–98.

Shapiro, R.: 1995, The Prebiotic Role of Adenine: A Critical Analysis, *Orig. Life Evol. Biosph.* **25**, 238–239.

Voet, A. B. and Schwartz, A. W.: 1983, Prebiotic Adenine Synthesis from HCN: Evidence for a Newly Discovered Major Pathway, *Bioorganic Chem.* **12**, 8–17.