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Alternative Bases in the RNA World: The Prebiotic Synthesis of Urazole and Its Ribosides

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Abstract. Urazole is a five-membered heterocyclic compound which is isosteric with uracil's hydrogen-bonding segment. Urazole reacts spontaneously with ribose (and other aldoses) to give a mixture of four ribosides: α and β pyranosides and furanosides. This reaction occurs in aqueous solution at mild temperatures. Thermodynamic and kinetic parameters for the reaction of urazole with ribose were determined. In contrast, uracil is completely unreactive with ribose under these conditions. Urazole's unusual reactivity is ascribed to the hydrazine portion of the molecule. Urazole can be synthesized from biuret and hydrazine under prebiotic conditions. The prebiotic synthesis of guanazole, which is isosteric in part to diaminopyrimidine and cytosine, is accomplished from dicyandiamide and hydrazine. Kinetic parameters for both prebiotic reactions were measured. Urazole and guanazole are transparent in the UV, which would be a favorable property in the absence of an ozone layer on the early Earth. Urazole makes hydrogen bonds with adenine in DMSO similar to those of uracil, as established by ^{1}H NMR. All of these properties make urazole an attractive potential precursor to uracil and guanazole a potential precursor to cytosine in the RNA or pre-RNA world.

Key words: Prebiotic synthesis — Urazole — Ribose — Urazole ribosides — Guanazole — Alternative nucleoside bases

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Introduction

It has become clear that RNA itself cannot be the first genetic material (Joyce et al. 1987). The reasons are many: ribose is unstable and cannot be made in adequate quantities by the formose reaction (Shapiro 1988); the prebiotic synthesis of pyrimidines is only fair compared to the excellent synthesis of purines; prebiotic nucleoside synthesis is poor with purines and does not occur with pyrimidines (Fuller et al. 1972a,b); and activated pyrimidines do not undergo template polymerizations on poly-purine templates (Joyce 1987). This paper attempts to address the nucleoside synthesis problem.

One place to look for potential replacements for the pyrimidines in the precursor to RNA is in the minor nucleosides of t-RNA. We are investigating the prebiotic syntheses of a number of these bases (Robertson et al. 1993), and several of them look as prebiotic as uracil and cytosine. However, it is possible that the first genetic material contained bases that are very different from those in t-RNA. A number of alternative base pairs have been shown to be incorporated enzymatically into DNA and RNA, but these base pairs are not prebiotic (Piccirilli et al. 1990).

A more prebiotic series of compounds is urazole, cytazole, and guanazole,[1] which are mimics of uracil

[1] The similarity of the names of urazole and guanazole to uracil and guanine is of interest. The first urazole synthesized was 1-phenylurazole from phenylhydrazine hydrochloride and urea (Pinner 1887). The parent compound was prepared by Pellizzari (1894) from hydrazine sulfate and biuret [and shortly after by Thiele and Stange (1894) by heating hydrazine dicarboxamide]. Similarly the first guanazole synthesized was 1-phenylguanazole from phenylhydrazine hydrochloride and dicyandiamide (Pellizzari 1891). The parent compound was prepared from hydrazine hydrochloride and dicyandiamide (Pellizzari 1894).

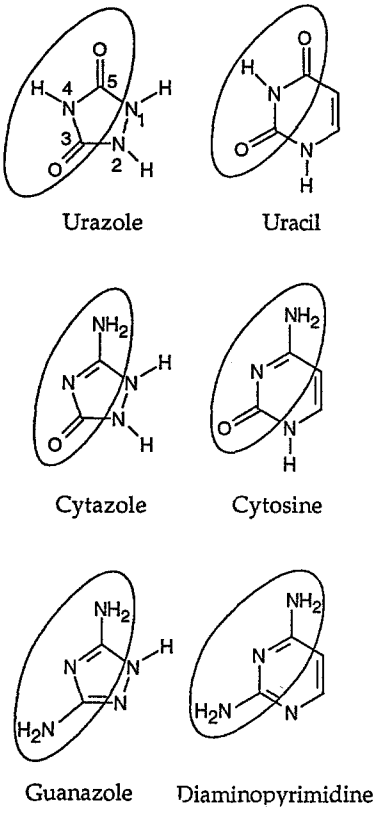


Fig. 1. Hydrogen bonding centers of uracil, urazole, and related compounds.

and cytosine (Fig. 1). Urazole is a five-membered heterocyclic compound that is isosteric in part to uracil; 5-amino-1,2,4-triazole-3-one (which we call cytazole) bears a similar relation to cytosine as urazole does to uracil. Guanazole is isosteric in part to diaminopyrimidine and cytosine. We show here that urazole and guanazole are potential prebiotic compounds. Urazole reacts rapidly with ribose to form a mixture of nucleosides with a favorable equilibrium constant. Urazole forms hydrogen bonds with adenine in DMSO in a manner similar to uracil-adenine.

Materials and Methods

1-Methylurazole was synthesized by the method of Bausch et al. (1991). Urazole, 4-methylurazole, 6-azauracil, 6-azauridine, uridine, D-ribose 5-phosphate disodium salt dihydrate, guanazole, biuret, and TSPS (3-trimethylsilyl propionic acid, sodium salt) were purchased from Aldrich. D-ribose was purchased from both Aldrich and Fisher. Dicyandiamide and hydrazine hydrate were from Fisher. All deuterated solvents were from Cambridge Isotope Laboratories.

NMR analysis was done on a GE QE-300 NMR with a 5-mm broad band and devoted ^1H and ^{13}C probe. HPLC analysis was with two Beckman 110B pumps controlled by Beckman System Gold and analyzed by a Kratos Spectroflow 757 UV detector set to 232 nm. Riboside separation was achieved with an Alltech HEMA-IEC BIO 1000 Q 10U (10 × 250 mm) strong anion exchange preparative column. The solvent system was 2.0 mM NaOH at a flow of 4 ml/min. Guanazole was separated from dicyandiamide on an analytical-scale HEMA column (4.6 × 150 mm) with water as the mobile phase at 1.5 ml/min. Urazole was separated from biuret under the same scheme as guanazole and dicyandiamide but at 220 nm. UV/Vis analysis was done on an HP 8452A diode-array spectrophotometer. Supracil UV quartz micro-cells (0.5 ml) were used.

Reaction temperatures were held constant (±0.1°C as measured by a quartz thermometer) in dry baths (at 120°C, 100°C, 80°C, or 60°C) or in thermostated water baths regulated by thermostated circulating heaters (at 40°C or 25°C).

Reaction of Urazole and Related Compounds with Ribose or Other Sugars. Urazole and D-ribose (0.4 M) were dissolved under mild heating in D_2O. The mixture was heated at various temperatures and times. The progress of the reaction was monitored by ^1H NMR. The products were separated via preparative-scale HPLC, and the individual peaks were characterized via ^1H and ^{13}C NMR; 4-methylurazole, 1-methylurazole, 6-azauracil, and guanazole were treated similarly.

Urazole (0.32 M) was dissolved with equimolar D-ribose 5-phosphate, as disodium salt dihydrate, in warm D_2O (2.5 ml). The mixture was heated just below reflux for 2 days. A yellow-colored solution and an insoluble precipitate were generated. The former was subjected to an NMR analysis.

Synthesis of Urazole and Guanazole. Urazole was synthesized by heating of hydrazine hydrate and biuret in a sealed tube. The reaction of 2.3 M hydrazine and 0.26 M biuret was followed at 80°C to completion. A kinetic study of this reaction was done with 1.1 M hydrazine and 0.13 M biuret at 120°, 100°, 80°, 60°, and 40°C. In addition, the reaction was observed at 80°C with 0.26 M biuret while the hydrazine concentration was varied from 0.06 to 2.3 M. Reactions were stopped by removing the tubes from the heating blocks. The samples were dried under vacuum, dissolved in DMSO-d_6, and analyzed via ^{13}C NMR.

The reaction of dicyandiamide with hydrazine to form guanazole was studied at 100°, 80°, 60°, 40°, 25°, and 4°C, with 1 M dicyandiamide and 2.3 M hydrazine. At 100°C hydrazine concentrations were varied from 0.09 to 2.3 M.

Results

Products of Urazole and Ribose and Related Reactions

Four ribosides were separated by HPLC and identified by ^1H and ^{13}C NMR (Tables 1, 2). These correspond to the α and β furanosides (F) and pyranosides (P) (Fig. 2). The initial products are 13% α-F, 22% β-F, 11% α-P, and 53% β-P. The equilibrium products are 5% α-F, 9% β-F, 8% α-P, and 78% β-P. These differ from the ribose values of 6% α-F, 11% β-F, 20% α-P, and 63% β-P (Table 3). Kinetics of the interconversions will be investigated subsequently. The products were identified by comparing the chemical shifts of the unknown peaks with model compounds (ribose, uridine, 6-azauridine, ribosyl amines, and pyranosylaminoguanidines). C-1′ shifts for α and β pyranosyl ribosides are very similar, while the α and β furanosyl ribosides are about 5 ppm apart (β-F being more downfield). The same trend is seen in the ribose isomers. The C-4′ shift in ribose is 70 ppm for pyranoses and 85 ppm for furanoses. Similar differences for the C-4′ shifts of the

Table 1. ^{1}H NMR chemical shifts of urazole ribosides (δ, ppm)[a]

Isomer	H-1	H-2	H-3	H-4	H-5
β-P	5.19	3.98	4.24	3.84–3.92	3.66–3.78
	(d; J = 9.4 Hz)[b]	(d,d; J = 9.3,2.5 Hz)	(s)	(m)	(m)
α-P	5.28	3.96–3.97[c]	4.22	3.96–3.97	3.68–3.84
	(d; J = 2.2 Hz)	(m)	(s)	(m)	(m)
β-F	5.57	4.44	4.23	4.00–4.04	3.60–3.80
	(d; J = 6.0 Hz)[d]	(t; J = 5.7 Hz)	(t; J = 4.8 Hz)	(m)	(m)
α-F	5.88	4.51	4.26–4.31	4.04–4.06	3.60–3.80
	(d; J = 6.7 Hz)	(t; J = 6.7 Hz)	(m)	(m)	(m)

[a] D_2O, TSPS as an internal standard or TMS in $CDCl_3$ as an external standard
[b] In agreement with reported range for cyclic β-P of aldose guanylhydrazones (Szilágyi et al. 1986)
[c] H-2 and H-4 signals overlap
[d] 5.52 (d, J = 5.0 Hz) was reported (Witkowski and Robins 1970) for the urazole β-F riboside

Table 2. Comparison of ^{13}C NMR chemical shifts of ribose and its ribosides[a]

	Ribose[b]				Uridine[c]	6-Azauridine[d]	Urazole ribosides[e]			
Carbon	α-F	β-F	α-P	β-P	β-F	β-F	α-F	β-F	α-P	β-P
C-1′	99.00	103.65	96.25	96.55	92.18	92.62	87.54	93.24	79.89	80.23
C-2′	73.70	77.98	72.75	73.75	72.24	72.95	68.46	70.69	69.42	70.14
C-3′	72.75	73.15	71.95	71.70	76.53	75.63	74.03	74.41	67.31	66.32
C-4′	85.76	85.21	70.00	69.95	87.03	86.83	83.39	83.47	66.65	65.39
C-5′	65.24	64.09	65.72	65.72	63.56	64.27	60.47	60.86	62.39	63.89
C-2					154.47	152.93				
C-3							154.23	155.21	154.42	155.04
C-4					169.01	161.72				
C-5							154.86	156.25	154.46	155.97

[a] D_2O, TSPS as an internal standard, or TMS in $CDCl_3$ as an external standard
[b] Breitmeier and Hollstein (1976)
[c] Chang et al. (1982)
[d] Jones et al (1970)
[e] Peak assignment by analogy with those of ribose anomers[b], uridine[c], 6-azauridine[d], ribosyl amines, Chavis et al. (1983) and pyranosylaminoguanidines, Szilágyi et al. (1986)

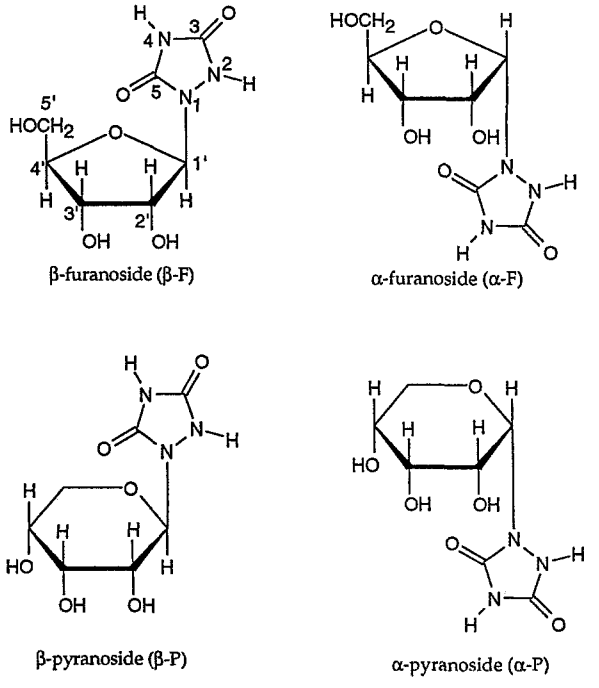


Fig. 2. Structures of urazole ribosides

pyranose and furanose forms are observed for the urazole ribosides. The β-F shifts are analogous to those of uridine and 6-azauridine, as expected. The values are given in Table 2. Assignments for all isomers are also in general agreement with a series of ribosylamines (Chavis et al. 1983).

No open-chain hydrazones could be detected, as indicated by the lack of the characteristic C=N shift at 140–150 ppm. Such open-chain forms were observed for ribose hydrazones (Williams 1983) and for guanylhydrazones of a series of sugars (Szilágyi et al. 1986).

Urazole gave only α and β furanosides when treated with D-ribose-5-phosphate since pyranosides cannot be formed. The rate was approximately the same as with ribose, as was the α-F/β-F ratio.

Urazole also reacts with other aldoses to give various aldoside isomers. The sugars include allose, arabinose, galactose, glucose, lyxose, mannose, and xylose. These results will be reported separately.

4-Methylurazole reacts with ribose to give very similar results to those of the reaction with urazole. In contrast, 1-methylurazole does not react with ribose, even upon prolonged heating (over 5 months at 80°C). No ri-

Table 3. Change of urazole ribosides composition with time at 25°C[a]

	α-F	β-F	α-P	β-P
1 day	13%	22%	11%	53%
23 days	6%	10%	12%	72%
36 days	5%	8.5%	8.5%	78%
86 days	5%	9%	8%	78%

[a] The urazole ribosides were synthesized with a concentrated solution (10 M) solution and paste at 100°C. The sample was diluted by a factor of 10 and left at 25°C

bosides or other urazole products were observed by NMR. The combined results of the reactions of 4- and 1-methylurazole with ribose were also used to assign the point of ribose attachment to urazole at the hydrazine nitrogen. Also, the ^{13}C-NMR C=O chemical shifts of the urazole ribosides indicate an unsymmetrically substituted urazole (Table 2). 6-Azauracil was unreactive with ribose, since it gave no products at 80°C over a period of 7 months. Guanazole reacted with ribose to give a mixture of riboside products, some of which appear to be analogous to those of the reaction of urazole and ribose. These products are being investigated.

Base Pairing Between Urazole and Adenine

The evidence for specificity of base pairing between urazole and adenine was sought by following the ^{1}H-NMR method of Shoup et al. (1966). Hydrogen bonding was measured as a downfield shift of the N-H protons of 9-ethyladenine, 1-methyluracil, and 1-methylurazole. Our data, together with that of the literature, are presented in Table 4. The data show considerable hydrogen bonding between 1-methylurazole and 9-ethyladenine, comparable to that between 1-methyluracil and 9-ethyladenine.

Rate of Reaction of Ribose with Urazole

Ribose reacts readily with urazole at elevated temperatures under relatively concentrated conditions. The reaction is first order in urazole and first order in ribose (0.1 to 10 M) (data not shown). The rate is approximately independent of pH between pH 3 and 7 (data not shown). The kinetics were not investigated outside this range because of the difficulty in buffering the solutions, and because of the decomposition of the ribose.

Figure 3 shows the second-order rate constants as a function of temperature. The line corresponds to $\Delta H^{\ddagger} = 18.25 \pm 0.30$ kcal.

$$\log k\,(\mathrm{M^{-1}s^{-1}}) = 6.43 - \frac{3988}{T}$$

The half-life with 1 M ribose is 70 days at 25° and 3.2 years at 0°C.

Table 4. N-H NMR Chemical shifts[a] of various base combinations

	Proton (ppm)			
Base	NH	NH_2	Δ(A + U)	Δ(A + Uz)
9-ethyladenine (A)		7.178	0.017 (0.02)[b]	0.008
1-methyluracil (U)	11.216		0.058 (0.05)[b]	
A + U	11.274	7.195		
1-methylurazole (Uz)	10.529			0.031
A + Uz	10.560	7.186		

[a] All concentrations are 0.1 M in DMSO-d_6
[b] Shoup et al. values (1966)

Equilibrium Constant

The equilibrium constant for the reaction

$$\text{Ribose} + \text{Urazole} = \text{Urazole-riboside}$$

$$K_{eq} = \frac{[\text{Urazole-riboside}]}{[\text{Ribose}][\text{Urazole}]}$$

was measured directly, approaching the equilibrium from both sides. The urazole-riboside refers to the sum of the four isomers. The approach from the riboside-poor side was followed by starting with ribose and urazole. The approach from the riboside-rich side was accomplished by heating a 0.4 M ribose + 0.4 M urazole solution at 40°C to about 20% completion. This mixture of the four anomers is riboside rich at 60°, 80°, and 100°C. The loss of riboside at the desired temperature was then followed by NMR. Figure 4 shows the plot of the K_{eq} at 40° (0.90), 60° (0.63), 80° (0.50), and 100° (0.425). The Van't Hoff plot is linear and is given by

$$\log K_{eq} = -2.08 + \frac{633.7}{T}$$

$$\Delta H = -2.9 \pm 0.3 \text{ kcal}$$

Prebiotic Synthesis of Urazole and Guanazole

Urazole and guanazole are very simple compounds which look potentially prebiotic. There are many potential prebiotic routes, but the most promising for urazole is the thermal synthesis from biuret (Pellizzari 1894). Biuret is easily synthesized from urea by heating, and urea is considered to be a major prebiotic compound. The reaction is

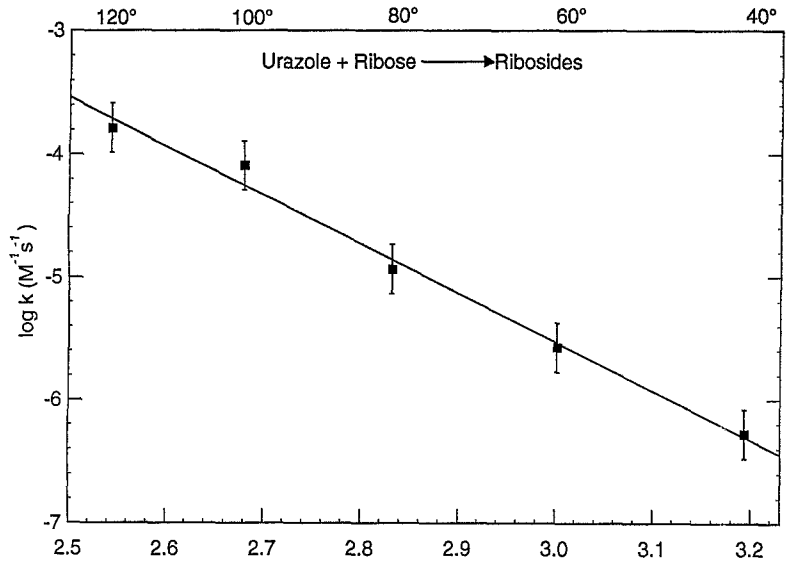


Fig. 3. Arrhenius plot of the second-order rate constants for the reaction of urazole and ribose to give the ribosides.

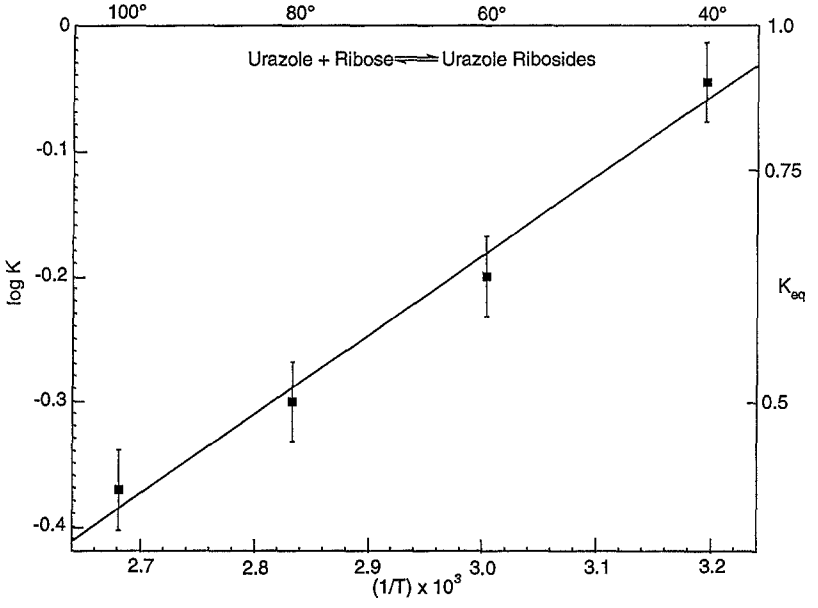
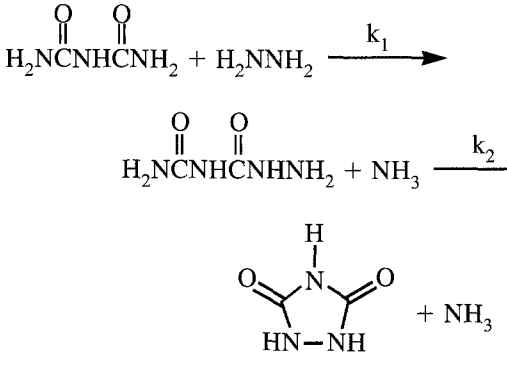


Fig. 4. Van't Hoff plot of the equilibrium constant for the formation of urazole ribosides.



The Pellizzari synthesis is the heating of biuret and hydrazine without solvent at 130°C for several hours. A lower-temperature route would be more prebiotic, so we investigated the reaction in aqueous solution at lower temperatures. The reaction was followed by ^{13}C NMR, with the peaks calibrated by known biuret and urazole (155.6 ppm and 156.5 ppm, respectively, in DMSO-d_6). The reaction is first order in both biuret and hydrazine. The Arrhenius plot is shown in Fig. 5. The heat of activation is 14.5 kcal. The half-lives with 1 M hydrazine at 25° and 0°C are 43 days and 403 days, respectively.

$$\log k = 3.90 - \frac{3{,}170}{T}$$

The slow step in this reaction is the addition of hydrazine to biuret, followed by a ring closure to urazole. Urea and semicarbazide were also observed (159.9 ppm and 161.4 ppm, respectively, in DMSO-d_6). These can be accounted for on the basis of loss of NH_3 from the tetrahedral intermediate in one case and loss of urea in the second case.

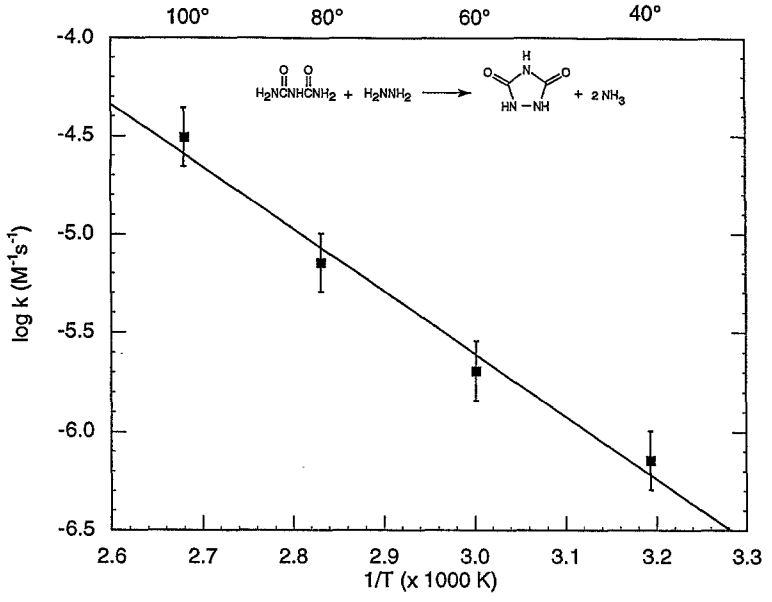


Fig. 5. Arrhenius plot of the second-order rate constants for the reaction of biuret and hydrazine to give urazole.

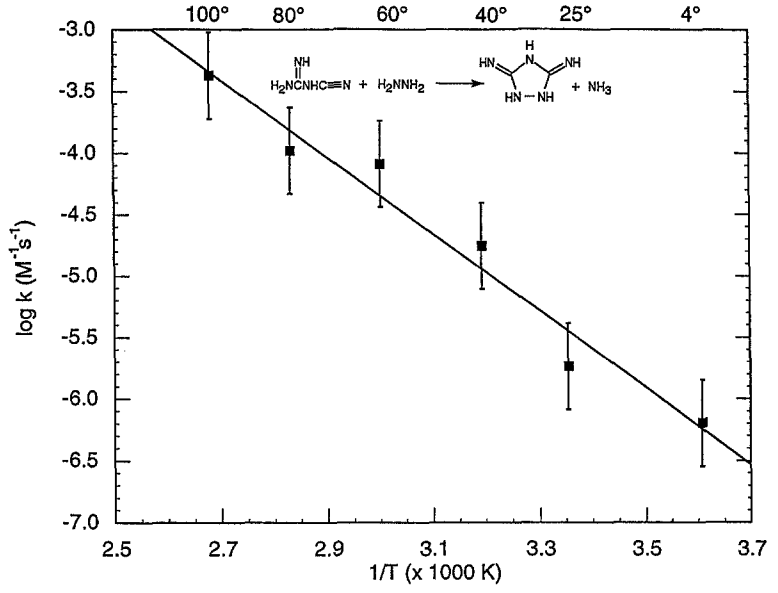
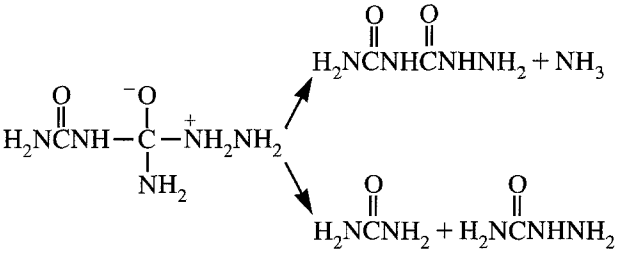
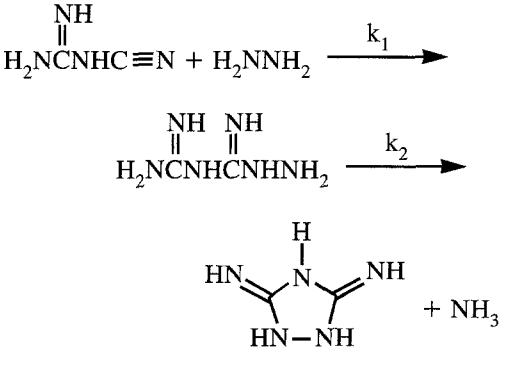


Fig. 6. Arrhenius plot of the reaction of dicyandiamide and hydrazine to form guanazole.



The yield of urazole is 35% at 80°C (5 days) with 2.2 M hydrazine and 0.13 M biuret. The yield was higher (51%) at 40°C for 164 days. Semicarbazide, urea, and a small amount (<5%) of unidentified compounds (162.0 and 159.8 ppm) account for the rest of the products.

The reaction of hydrazine with the dimer of cyanamide, dicyandiamide, is a potential prebiotic synthesis of guanazole.



Cyanamide is an accepted prebiotic compound (Miller and Orgel 1974), which easily dimerizes to dicyandiamide (Hammett 1940). The guanazole synthesis reaction is first order in both dicyandiamide and hydrazine (data not shown). The Arrhenius plot is shown in Fig. 6. The heat of activation is 17.4 kcal. The rate half-lives with 1 M hydrazine are 54 h and 20 days at 25° and 0°C, respectively.

$$\log k = 5.04 - \frac{3{,}130}{T}$$

The reaction was followed by ^{13}C NMR by observing peaks for dicyandiamide (163.3 ppm C=N; 119.2 ppm C≡N) and guanazole (158.7 ppm).

The rate constant at 25°C for the reaction is 19 times that of the biuret-hydrazine reaction. This is attributed to hydrazine being an α-effect nucleophile whose enhanced reactivity is particularly marked with nitriles (Grekov and Veslov 1978; Fina and Edwards 1973).

The yield of guanazole is 32% at 80° (16 h) with 2.2 M hydrazine and 1 M dicyandiamide. The yield is higher (43%) at 25° in 28 days. The unidentified compounds at 162.5, 161.9, 160.5, and 151.5 ppm comprise the rest of the material.

Discussion

It is generally accepted that an RNA world would exist before the origin of protein synthesis, but it is equally clear that there must have been a precursor to RNA. The central problem is the precursor to the ribose phosphate backbone, but an important issue is the bases used. There is no reason to believe that they were AUGC, and a number of alternatives have been proposed. Urazole and guanazole have not been previously considered as prebiotic compounds and they are very different from AUGC or the other proposed alternatives. We believe that our results with urazole and guanazole suggest that the search for precursors to AUGC should not be confined to purines and pyrimidines: other heterocyclic systems that can form stable base pairs should be considered.

Hydrazine is generally omitted in prebiotic schemes because it is relatively unstable thermodynamically and because of the rare occurrence of the N-N bond in biology. This omission is unnecessary since there are several plausible prebiotic syntheses of hydrazine and hydrazine-containing molecules. These include the low-yield syntheses from the photolysis of NH_3 + CO (Ferris et al. 1974) and by the action of a spark discharge on aqueous N_2 + $CaCO_3$ (Folsome et al. 1981). The yield is higher from an electric discharge with NH_3 (Thornton and Spedding 1967), but the best synthesis of hydrazine is probably the reaction of traces of ClO^- with NH_3 (Raschig Process) (Schmidt 1984). This is an efficient process of commercial importance, and its prebiotic possibilities need to be investigated.

The equilibrium constant of about 1.0 for the formation of the urazole riboside is far more favorable than that for the formation of uridine.

$$\text{Uracil} + \text{Ribose} = \text{Uridine} + H_2O \qquad K_{eq} = 10^{-3}$$

This value comes from (Bose and Yamada 1974; Pontis et al. 1961; Camici et al. 1980)

$$\text{Uracil} + \text{Ribose-1-phosphate} = \text{Uridine} + P_i \qquad K = 10$$

$$\text{Ribose} + P_i = \text{Ribose-1-phosphate} \qquad K = 1.0 \times 10^{-4}$$

This is a major consideration for the prebiotic synthesis of nucleosides since the unfavorable equilibrium constant means that the ribose needs to be activated or the mixture of uracil and ribose needs to be heated to reduce the activity of water. However, this does not work with pyrimidines and gives only low yields with purines (Fuller et al. 1972b). Urazole riboside synthesis by contrast would be possible in a dried-up lake bed or with evaporated sea water, provided ribose was available and concentrated. The formation of urazole riboside would not take place in the open ocean because the equilibrium is not sufficiently favorable.[2]

It is generally felt that ribose is not a realistic component of the ribose phosphate precursor because the formose reaction would produce too many interfering sugars and because of stability problems with all sugars (Shapiro 1988). Since the equilibrium constant for urazole is ~1,000 times more favorable than with uracil, it would be expected that the unknown ribose precursor equilibrium constant would also be about 1,000 times more favorable. Similarly, the rate constants for the reaction with the ribose precursor would be expected to be more favorable with urazole than with uracil, unless some special mechanism is involved. Thus urazole will always be more favorable than uracil as a prebiotic base in its equilibrium and rate of reaction with the pre-RNA backbone.

A remarkable feature of urazole and guanazole is their transparency in the ultraviolet region of the spectrum. These spectra are shown in Fig. 7. Urazole exhibits its maximum at 204 nm (ε = 6,680) and guanazole absorbs at 206 nm (ε = 7,890), whereas uracil has its maximum at 259 nm. The molar absorptivities (ε) at 260 nm are 127 for urazole, 148 for guanazole, and 7,720 for uracil.

[2] It has been reported that ribose-5-phosphate reacts spontaneously with barbituric acid to form a phosphodiester bond with the 2-oxygen of barbituric acid (Komura et al. 1980). It is more likely that this reaction takes place at the 5-carbon (Gonzales et al. 1986)

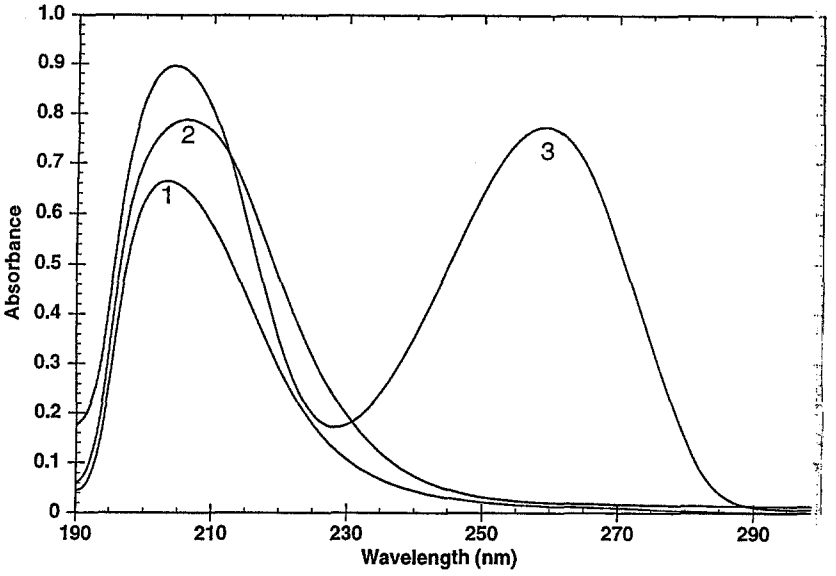


Fig. 7. Comparison of the absorbance of urazole (1) and guanazole (2) with that of uracil (3). Each compound was 99 mM and analyzed individually in pH 6 phosphate buffer.

Unlike AUGC, the transparency of urazole and guanazole in the ultraviolet would result in nucleic acids containing these bases being protected from ultraviolet degradation on the early Earth. The ultraviolet flux at the Earth's surface is thought to have been much higher than at present because of the absence of an ozone layer. The ultraviolet transparency of urazole and guanazole would be a strong selective advantage for such nucleic acids, unless there were strong ultraviolet absorbers in the environment to protect ultraviolet-absorbing nucleic acids.

There is no obvious choice for nonultraviolet-absorbing complements to urazole and guanazole. It is possible that adenine and guanine were used with urazole and guanazole because these purines are less damaged by ultraviolet than pyrimidines (Elad 1976). In addition, they seem to be among the most abundant prebiotic heterocyclic bases.

Urazole forms a mixture of α and β furanosides and pyranosides with ribose (and other pentoses and hexoses). Equilibration of these forms was studied for ribose. The original mixture, in which the β-P form is the predominant one (53%) and β-F is second in abundance (22%), changes upon prolonged standing to a mixture in which the β-P is even more prominent (78%), while the β-F form is diminished (9%). The ribopyranose form has been indicated as potentially prebiotically important in pre-RNA due to the considerable stability it gives to the pyranose-RNA, as pointed out by Eschenmoser (Pitsch et al. 1993). The kinetic and thermodynamic predominance of the P form of the urazole ribosides may be of prebiotic importance. It should be noted that traditional nucleosides—for example, uridine—exist solely in the β-F form, which does not equilibrate to any observable amount (by NMR) over a period of several months.

The results reported here show that there are potential RNA precursors to purines and pyrimidines in general and to AUGC in particular. Some hydrazine-containing alternatives are attractive, and hydrazine needs to be further considered as a prebiotic reagent.

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