ATTEMPTED PREBIOTIC SYNTHESIS OF PSEUDOURIDINE

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Abstract. Pseudouridine is a modified base found in all tRNA and rRNA. Hence, it is reasonable to think that pseudouridine was important in the early evolution, if not the origin, of life. Since uracil reacts rapidly with formaldehyde and other aldehydes at the C-5 position, it is plausible that pseudouridine could be synthesized in a similar way by the reaction of the C-5 of uracil with the C-1 of ribose. The determining factor is whether the ribose could react with the uracil faster than ribose decomposes. However, both rates are determined by the amount of free aldehyde in the ribose. Various plausible prebiotic reactions were investigated and none showed pseudouridine above the detection limit (<0.01%). Only unreacted uracil and ribose decomposition products could be observed. Thus the rate of addition of ribose to uracil is much slower than the decomposition of ribose under any reasonable prebiotic conditions. Unless efficient non-biological catalysts for any of these reactions exist, pseudouridine would not have been synthesized to any significant extent without the use of biologically produced enzymes.

Abbreviations: Ψ – pseudouridine; HMU – 5-hydroxymethyluracil; 6CMU – 6-carboxymethyluracil; R1P – ribose-1-phosphate; PRPP – 5-phosphoribosyl-1-pyrophosphate; 6MeU – 6-methyluracil; F – furanose; P – pyranose

1. Introduction

Pseudouridine (Ψ) is a modified base consisting of a ribose linked to the C-5 of uracil rather than the N-1 attachment of uridine. Ψ is found in various positions in transfer RNA (tRNA), including the highly conserved TΨC loop. In addition, several Ψ are found in ribosomal RNA (rRNA) and are believed to be important for peptide bond formation (Lane *et al.*, 1995). It therefore seems likely that Ψ played an important role in early evolution, and perhaps even in the origin of life.

Except for the synthesis of α cytosine (Sanchez and Orgel, 1970), the prebiotic synthesis of nucleosides has not been observed with pyrimidines and is not efficient for purines (Fuller, *et al.*, 1972a,b). The closest thing to a prebiotic pyrimidine nucleoside is the urazole riboside which is a mimic of uridine (Kolb *et al.*, 1994). The prebiotic synthesis of Ψ is important not only because it might have been an important co-factor, but it could have served as a substitute for uridine in early genetic material.

Since uracil reacts rapidly with formaldehyde and other aldehydes at the C-5 position (Robertson and Miller, 1995a), Ψ might be synthesized in a similar manner by the reaction of the C-5 of uracil with the C-1 of ribose. The equilibrium constant (K_1) for the formation of 5-hydroxymethyluracil (HMU) from uracil and

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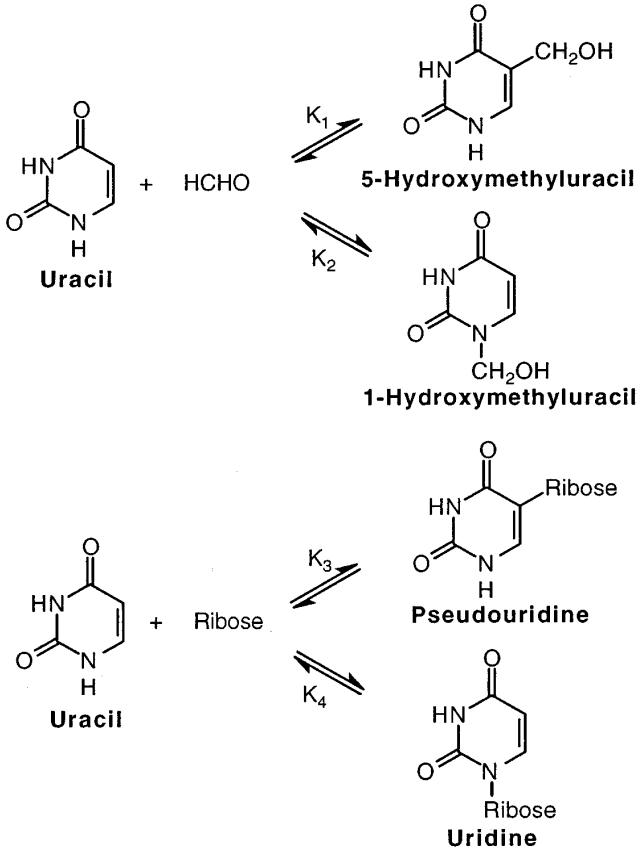
formaldehyde has been determined to be 45 000 at 25 °C (Robertson and Miller, 1995). The equilibrium constant (K_2) for the formation of 1-hydroxymethyluracil from uracil and formaldehyde has been measured as 1.0 at 20 °C (Lewin, 1964). The equilibrium constant (K_4) for the reaction of ribose with uracil to form uridine has been calculated to be about 10^{-3} (Kolb *et al.*, 1994).



If the ratio of the equilibrium constants of the reaction of uracil at the C-5 and N-1 positions with formaldehyde are proportional to their reaction with ribose,

$$\frac{K_1}{K_2} = \frac{K_3}{K_4} \tag{1}$$

then $K_3 \approx 45$. If the reaction is indeed reversible, it seems that it would be favorable.

The decomposition of Ψ to produce uracil has been measured at 100 °C to have $k = 2.7 \times 10^{-8} s^{-1}$. Taking the K_{eq} to be 45 and a k_r of $2.7 \times 10^{-8} s^{-1}$, since

$$K_{eq} = \frac{K_f}{K_r} \tag{2}$$

the pseudo-first-order rate constant for the formation of Ψ would be approximately $1.2 \times 10^{-6} s^{-1}$ (for 1 M ribose) that gives a $t_{1/2} \approx 6.6$ days. Thus, based on the principle of microscopic reversibility it would be expected that Ψ could be synthesized from ribose and uracil, provided the ribose does not decompose before it can react with uracil. However, Larralde *et al.* (1995) have reported a half-life of 73 minutes for the decomposition of ribose at 100 °C, pH 7, and 0.02 M phosphate. In addition, Shapiro (1988) has discussed several other shortcomings in the availability of ribose, which should be considered in this and other papers concerning the prebiotic synthesis of nucleosides.

Since the rate of decomposition of the nucleoside is so much slower than the decomposition of ribose, it is possible that detectable quantities of Ψ could be formed, especially where ribose is in great excess. This is feasible because of the high solubility of ribose (20 *m*) (Goldberg and Tewari, 1989) and deoxyribose (30 *m*) (determined using the NMR method of Keefe *et al.* (1995)). Nevertheless, in all conditions investigated, the ribose decomposed before enough Ψ formed to be detected.

2. Experimental

2.1. Materials and analytical method

D-Ribose, 2-deoxy-D-ribose, 6-carboxymethyluracil (6CMU), uracil, D-ribose-1-phosphate (R1P), 5-phospho-D-ribosyl-1-pyrophosphate (PRPP), sea salts, and dimethylamine were from Sigma. The argon and titanium dioxide were from Aldrich. The deuterium oxide and DMSO-d6 were purchased from Cambridge Isotope Laboratories. The orotic acid was obtained from Calbiochem. The Chemical Dynamics Corporation and Sigma supplied the α- and β-D-furano-pseudouridine. Magnesium chloride was from Mallinckrodt, and the sodium acetate and urea were from Fisher. The cyanoacetaldehyde was prepared from isoxazole as described by Ferris *et al.* (1968).

^{1}H NMR analysis was performed on a GE QE-300 NMR with a 5 mm broadband probe. HPLC analysis was performed with two Beckman 110B pumps controlled by Beckman System Gold and analyzed with a Kratos Spectroflow 757 UV detector set to 260 nm. Except where noted differently, pseudouridine separation was achieved with a YMC-Pack ODS-AQ S-5 μm (150 × 6 mm) reverse-phase column. The solvent system was isocratic pH 4.8 0.05 M sodium phosphate at a flow of 2 mL/minute.

Since the α- and β-pyranose and furanose (P and F) isomers of Ψ can interconvert, as is discussed by Chambers *et al.* (1963), the detection of any one isomer would demonstrate the existence of all of them. The HPLC elution system employed separates the α- and β-P and F isomers of Ψ (Figure 1a); however, the β-P and both F peaks are masked by the high concentration of uracil. Only the α-P-Ψ was well

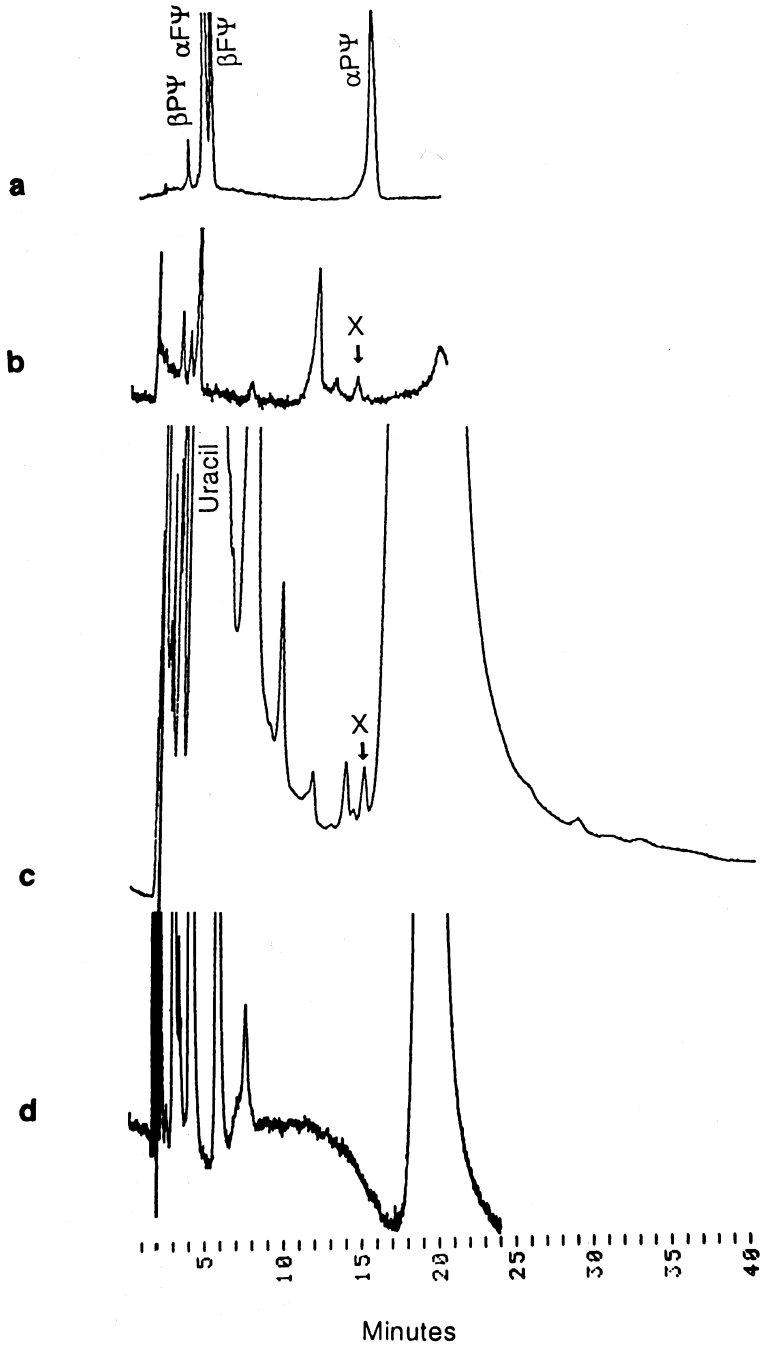


Figure 1. HPLC of: (a) α-P-Ψ which had been collected from isomerized β-F-Ψ and heated at 100 °C pH 4.8 for 12 hours; (b) HPLC of suspected α-P-Ψ (X) after it had been collected and heated at 100 °C pH 4.8 for 12 hours. Note that X does not actually line up with the α-PΨ above; (c) HPLC of 1 M ribose and 0.1 M uracil which had been heated at 60 °C for 77 days. X marks the location of suspected α-P-Ψ; (d) HPLC of 1 M ribose 80 °C pH 1 for 1 hour. Note that the large-scale features of this plot are the same as in 1c.

separated from uracil and most ribose decomposition peaks. Peaks were occasionally observed near the α-P-Ψ, but were demonstrated *not* to be Ψ because the peak, after it was collected and hydrolyzed, did not produce the four isomers as did an authentic sample of α-P-Ψ (Figure 1a,b,c). This was confirmed by a co-injection of the isomerized suspected peak with authentic Ψ which had been isomerized under identical conditions.

Reaction temperatures were held constant ($\pm$0.2 °C as measured by a quartz thermometer) in thermostatically-controlled dry baths (100, 80 and 60 °C), or in water baths regulated by thermostated circulating water held at 40 or 25 °C. The reaction at 170 °C was in an oil bath held at $\pm$1 °C.

2.2. Decomposition of pseudouridine

Solutions of 2×10^{-4} M α-F-Ψ or β-F-Ψ at pH 7 were heated to 100 °C in 0.02 M phosphate for up to 360 hours. They were analyzed with the following HPLC scheme: analytical HEMA IEC BIO 1000Q strong anion column with an isocratic mobile phase of 0.5 mm Tris·ClO_4 pH 12 at 1.5 mL/minute detected at 263 nm.

2.3. Attempted reproduction of previous work

Khenokh and Kuzicheva (1971) have reported a prebiotic synthesis of Ψ when 100 g of each of ribose, uracil, and TiO_2 were heated at 170 °C under argon for 0.5 or 2 hours. They reported a 4.5% maximum yield of Ψ as determined by paper chromatography (butanol-acetic acid-water (4:5:1)). The identity of their product was based on a comparison of acidic and basic UV spectra with literature values of Ψ and on the similarity of R_f value to uridine (not Ψ) on paper chromatography. Their experiment was repeated and the material was extracted with hot D_2O for analysis by ^{1}H-NMR, HPLC, and paper chromatography.

2.4. Effect of temperature and pH

The syntheses were attempted at 40, 60, 80 and 100 °C with 0.1 M uracil and 1 M ribose at an initial pH of either 7 or 8. The solutions were heated at the various temperatures from 30 minutes to 1 year. The pH of the solution quickly ($<$1 hour) decreases to about 4. It was not possible to buffer these reactions satisfactorily, as the ribose decomposition products are acidic and the high concentrations of buffer needed (such as phosphate) strongly catalyze ribose decomposition (Larralde *et al*, 1995). Since the reaction of formaldehyde with uracil is acid catalyzed, the above mixture at pH 0 and 1 was also heated at 80 ° for 1–24 hours without significant pH change.

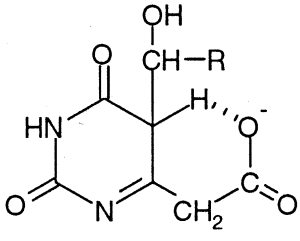
2.5. Effect of high ribose concentration

In an attempt to push the equilibrium in favor of Ψ, the ribose concentrations were increased. Solutions of 0.1 M uracil were heated at 60 °C with 1, 18, and 50 *m*

ribose* from 1 hour to 77 days. A 0.25 mL slurry of 10 *m* ribose and 10 *m* uracil was heated at 80 °C for up to 7 days, and 0.1 M uracil was dissolved by 0.1 mL of molten ribose (mp = 87 °C). This was then heated at 100 °C for 30 minutes.

2.6. USE OF A GENERAL BASE

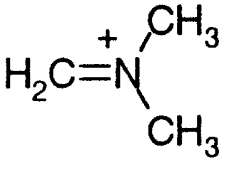
It has been observed that the rate of addition of formaldehyde to 6CMU and 6-methyluracil (6MeU) is faster than the addition to uracil (Robertson *et al.*, 1997). Presumably the rate of formaldehyde addition is faster for 6CMU because the acetate moiety forms a 6-membered ring with the carboxylate acting as an internal general base, and 6MeU adds HCHO faster because of electron donation.



Solutions of 0.1 M 6CMU and 1 M ribose were heated at 60 °C and 40 °C for up to 70 days. The detection was based primarily on ^{1}H NMR because of a lack of standards for HPLC. Since 6CMU decarboxylates to 6MeU ($k_{pH7,100\,°C} = 2.7 \times 10^{-8}$ s^{-1}) (Robertson *et al.*, 1997), the appearance of both pseudocarboxymethyluridine and pseudomethyluridine was monitored by ^{1}H NMR. An external general base was also tried, using 15 *m* sodium acetate heated (60 °C) with 1 M ribose and 0.1 M uracil for up to 70 days.

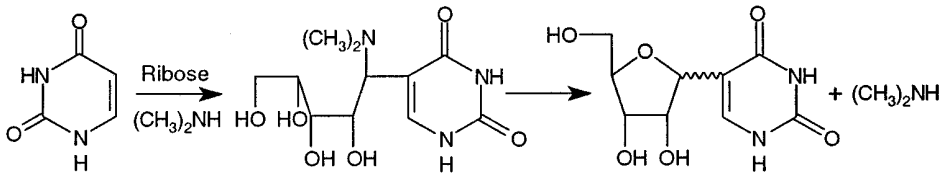
2.7. EFFECT OF A MANNICH AMINE AND Mg^{2+}

The Mannich reaction is an alternative route for the addition of formaldehyde to uracil that is more efficient than formaldehyde alone because the Mannich amine



* Molality (*m*) (moles solute/kg solvent) was used if the amount of solute exceeded the amount of water, or if a slurry rather than a solution was prepared.

is a good electrophile (Robertson and Miller, 1995; Robertson *et al.*, 1997). It was hoped that this route (e.g. to the furanosides):



would overcome the problems of ribose decomposition.

A solution of 0.1 M dimethylamine with 0.1 M uracil and 1 M ribose was heated at 80 °C for up to 60 days.

It is possible that the presence of divalent metals found in biological systems could be important for the synthesis of Ψ. A solution of 0.2 M $MgCl_2$, 0.1 M uracil, and 10 *m* ribose was heated at 80 °C for up to 60 days.

2.8. USE OF 2-DEOXYRIBOSE

Larralde *et al.* (1995) have observed that 2-deoxyribose decomposes 2.6 times more slowly than ribose. In addition, Dworkin and Miller (1997) have determined the free aldehyde content of deoxyribose to be 0.15% at 25 °C by ^{1}H NMR. Since there is much more free aldehyde in deoxyribose than in ribose (0.05% at 20 °C) (Angyal, 1984), it is likely that deoxypseudouridine could be formed more readily than Ψ. Solutions of 0.2 M uracil and 1 M deoxyribose were heated to 100 or 80 °C from 6 hours to 6 days. A slurry of 12 *m* deoxyribose and 10 *m* uracil was heated at 80 °C for 18 hours. A solution of 0.1 M uracil in 50 *m* deoxyribose was heated to 80 °C for up to 18 days. Each of these mixtures was analyzed by ^{1}H NMR.

2.9. USE OF DRYING CONDITIONS

The majority of the purine nucleosides synthesized in the drying conditions were 'unnatural' anomers (Fuller *et al.*, 1972a,b; Maurel and Convert, 1990). In the case of Ψ the presence of α-F and both pyranoses is not a problem prebiotically since the anomers can interconvert. In fact, a higher yield of α-P than β-F would aid their detection. Thus, a drying experiment similar to that of Fuller *et al.* (1972b) was attempted. Here, solutions of 0.15 M ribose or deoxyribose and 0.01 M uracil were dissolved in synthetic sea water. These solutions were 5-fold diluted with water, and then 5 mL of each were placed in petri dishes and evaporated to dryness in a 100 °C oven. They took 2.5 hours to dry. The solutions were redissolved in water and analyzed via HPLC.

2.10. Use of some biochemical intermediates

In an attempt to mimic a possible prebiotic or early biosynthetic pathway, uracil was mixed with R1P and PRPP. Solutions of 0.02 M uracil and either 1 M R1P or 1 M PRPP adjusted to pH 7.5 were allowed to react at 25 °C for 29 hours. In addition, solutions of 0.01 M uracil and 0.2 M R1P or 0.2 M PRPP, with or without 0.2 M $MgCl_2$, were allowed to react for 50 days at 25 °C. Also, saturated orotic acid (0.17 M at 60 °C and 0.094 M at 40 °C) and 1 M ribose were heated at 60 and 40 °C for up to 70 days. In addition, saturated orotic acid (0.064 M) and 1 M PRPP adjusted to pH 7.5 were allowed to react at 25 °C for 29 hours. The detection was based primarily on ^{1}H NMR because of a lack of standards for HPLC; however, the R1P reaction and any dephosphorylated product was monitored via HPLC.

Biologically, Ψ is synthesized by the rearrangement of uridine on the RNA polymer. Lane *et al*. (1995) have suggested that a sterically driven uridine to Ψ might occur abiotically. A solution of 0.01 M uridine at pH 8 was heated at 100 °C for one month.

2.11. In situ synthesis of uracil

It is possible that conditions exist where the uracil could be assembled on ribose to make Ψ. One set of these conditions was explored based on the high yield prebiotic synthesis of uracil by Robertson and Miller (1995b). A solution of 20 *m* urea, 1 *m* ribose, and 0.01 M cyanoacetaldehyde was adjusted to pH 8 and heated to 100 °C for 3 and 7 days.

3. Results

3.1. Decomposition of pseudouridine

The decomposition of Ψ produces an HPLC peak that corresponds to uracil, as determined with a co-injection. The first-order rate constant was calculated to be $2.7 \times 10^{-8} s^{-1}$.

3.2. Attempted reproduction of previous work

The procedure of Khenokh and Kuzicheva was repeated and no Ψ was detected by HPLC (<0.01%). In addition, under the paper-chromatographic conditions used by Khenokh and Kuzicheva, all four isomers of Ψ co-elute in a spot (R_f = 0.338) significantly different from either uridine (R_f = 0.507) or uracil (R_f = 0.612). The reaction product produced a spot on the paper (R_f = 0.615) consistent with uracil, and no other spots could be seen. ^{1}H NMR analysis showed large quantities of ribose decomposition products, but no Ψ (<2%) and very little ribose.

Table I
Summary of the results

Experimental section	Expected product	Detection	Yield
Reproduction of previous work	pseudouridine	HPLC	$<0.01\%$
Temperature and pH	pseudouridine	HPLC	$<0.01\%$
High ribose concentration	pseudouridine	HPLC	$<0.01\%$
General base catalysts	pseudocarboxymethyluridine	^{1}H NMR	$<2\%$
	pseudomethyluridine	^{1}H NMR	$<2\%$
	pseudouridine	HPLC	$<0.01\%$
Mannich amine and Mg^{2+}	pseudouridine	HPLC	$<0.01\%$
2-Deoxyribose	2-deoxypseudouridine	^{1}H NMR	$<2\%$
Biochemical Intermediates	pseudouridine	HPLC	$<0.01\%$
	pseuoduridine-5′-phosphate	^{1}H NMR	$<2\%$
	pseuodorotidine	^{1}H NMR	$<2\%$
	pseuodorotidine-5′-phosphate	^{1}H NMR	$<2\%$
In situ synthesis	pseudouridine	HPLC	$<0.01\%$

3.3. Other attempted syntheses of pseudouridine and related compounds

The results of the various attempts to synthesize Ψ are summarized in Table I.

When potential catalysts were used to aid the reaction, particularly in the case of dimethylamine, it was found that they catalyze ribose decomposition faster than they might have catalyzed Ψ production. It is important that the catalyst increase the rate of addition of ribose to uracil but not catalyze the decomposition of ribose. This is likely to be difficult as both the decomposition of ribose and the reaction of uracil with HCHO are general-base catalyzed (Larralde *et al*., 1995; Robertson *et al*., 1996).

While no Ψ was detected ($<0.01\%$) with the *in situ* method, it is conceivable that a synthesis of Ψ (or related compounds) using this approach might work under more specialized conditions. Other researchers have had some success in synthesizing other pyrimidine nucleosides (though not necessarily under prebiotic conditions) on arabinose (Sanchez and Orgel, 1970 and Smith *et al*. 1972, for example). In addition, it is possible that some Ψ could be formed by coupling a prebiotic synthesis of uracil with one of ribose (e.g. cyanoacetaldehyde, urea, formaldehyde). However, it is unlikely that the yield would be above the detection limit, due to the dominance of side reactions (e.g. the formation of urea-formaldehyde polymer).

4. Discussion

While it is not possible to test all possible reaction conditions, one can conclude from these results that Ψ was not significantly present on the early Earth, except perhaps under very specialized conditions or with an unstated catalyst.

The primary difficulty seems to be the stability of ribose. It is possible that Ψ could have been synthesized on a protected form of ribose or some other sugar which was then converted into the ribonucleotide and used in the elongation of RNA either prebiotically or using early enzymes. In a preRNA world which formed its informational and catalytic polymers from something other than ribose, it is possible that analogs of Ψ existed. In addition, if Ψ or a non-ribose analog could be synthesized, it would very rapidly isomerize into its α- and β-F and P forms. It has been suggested that the availability of an isomerizable nucleoside could be of benefit as a way of avoiding annomeric cross-inhibition problems in a growing polymer (Kolb *et al*., 1994). Thus, as insight is gained into the nature of the first informational macromolecule, it would be useful to determine if Ψ analogs of it can be readily formed.

It seems likely that the use of Ψ must be reserved for a period in biological history where RNA or protein enzymes were efficient enough to overcome the difficulties involved in the synthesis of Ψ. Ribose synthesis requires enzymes, and Ψ requires enzymes; whether these require protein enzymes or can be catalyzed by ribozymes remains to be determined.

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References

Angyal, S. J.: 1984, *Adv. Carbohydr. Chem. Biochem.* **42**, 15–68.
Chambers, R. W., Kurkov, V. and Shapiro, R.: 1963, *Biochemistry* **2**, 1192–1203.
Dworkin, J. P. and Miller, S. L.: 1997, *Origin Life Evol. Biosphere* **26**, 412–413.
Ferris, J. P., Sanchez, R. A. and Orgel L. E.: 1968, *J. Mol. Biol.* **33**, 693–704.
Fuller, W. D., Sanchez, R. A. and Orgel L. E.: 1972, *J. Mol. Biol.* **67**, 25–33.
Fuller, W. D., Sanchez, R. A. and Orgel L. E.: 1972, *J. Mol. Evol.* **1**, 249–257.
Goldberg, R. N. and Tewari, Y. B.: 1989, *J. Phys. Chem. Ref. Data* **18**, 809–880.
Khenokh, M. A. and Kuzicheva E. A.: 1971, *Dokl. Akad. Nauk SSSR* **197**, 464–466.
Keefe, A. D., Newton, G. L. and Miller, S. L.: 1995, *Nature* **373**, 683–685.
Kolb, V. M., Dworkin, J. P. and Miller, S. L.: 1994, *J. Mol. Evol.* **38**, 549–557.
Lane, B. G., Ofengand, J. and Gray, M. W.: 1995, *Biochimie* **77**, 7–15.
Larralde, R., Robertson, M. P. and Miller, S. L.: 1995, *Proc. Nat. Acad. Sci. USA* **92**, 8158–8160.

Lewin, S.: 1964, *J. Chem. Soc*. 792–809.
Maurel, M. C. and Convert, O.: 1990, *Origin Life Evol. Biosphere* **20**, 43–48.
Robertson, M. P., Dworkin, J. P. and Miller, S. L.: 1997 (in prep.).
Robertson, M. P. and Miller, S. L.: 1995, *Science* **268**, 702–705.
Robertson, M. P. and Miller, S. L.: 1995, *Nature* **375**, 772–774.
Sanchez, R. A. and Orgel L. E.: 1970, *J. Mol. Biol.* **47**, 531–543.
Shapiro, R.: 1988, *Origin Life Evol. Biosphere* **18**, 71–85.
Smith, C. V. Z., Robins, R. K. and Tolman, R. L.: 1972, *J. Org. Chem.* **37**, 1418–1422.