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Hydrolysis of Dihydrouridine and Related Compounds

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ABSTRACT: Dihydrouridine is absent from the tRNA of almost all hyperthermophiles and most Archaea but is ubiquitous in the tRNA of Eubacteria and Eukaryotes. In order to investigate whether this could be due to instability, the rate of ring opening of dihydrouridine was measured between 25 and 120 °C. The dihydrouridine ring is stable at 25 °C, but the half-life at 100 °C and pH 7 is 9.1 h, which is comparable to the doubling time of hyperthermophiles. This suggests an explanation for the absence of dihydrouridine from the tRNA of hyperthermophiles. The rates of ring opening of dihydrouracil, dihydrothymine, and 1-N-methyldihydrouracil were measured at 100 °C and pH 6−9, as were the equilibrium constants for ring closure of the ureido acids to the dihydrouracils. The pH rate profiles for ring opening and ring closing were calculated from the data. Possible roles for dihydrouracils in the pre-RNA world are discussed.

Dihydrouridine (DHUR)[1] is a post-translational modified nucleotide found in tRNA and reported in rRNA, snRNA, and chromosomal RNA (Limbach *et al.*, 1994). DHUR is ubiquitious in the tRNA of Eubacteria and Eukaryotes but is rarely found in the tRNA of Archaea. DHUR is absent from almost all hyperthermophiles (organisims growing above 80 °C), which are all Archaea with one exception (Best, 1977; Björk, 1986; Gupta & Woese, 1980; McCloskey, 1986; Edmonds *et al.*, 1991). It has been suggested that the absence of DHUR in hyperthermophiles may be due to its nonaromatic ring and weak stacking energy (Edmonds *et al.*, 1991). An additional factor may be its instability to ring opening and subsequent hydrolysis of the glycosidic bond, followed by breaking of the ribose phosphate bond. DHUR is known to be unstable, with base catalyzing the opening of the ring and acid hydrolyzing off the ribose (Levene & La Forge, 1912; Cohen & Doherty, 1956; Green & Cohen, 1957; Lindahl 1967). It has been suggested that most of the thermal inactivation of tRNA is caused by the decomposition of DHUR (Lindahl, 1967). In order to determine whether the rate of decomposition of DHUR at elevated temperatures can rationally explain the absence of DHUR in the tRNA of hyperthermophiles, we have measured the rate and equilibrium of ring opening of DHUR.

We were also interested in the stability of dihydrouracil (DHU) and DHUR because they are potential bases in the RNA world as well as potential prebiotic precursors to uracil (Chittenden & Schwartz, 1976; Harada *et al.*, 1978). The role of DHUR in tRNA is not clear. Poly(dihydrouridine) does not hydrogen bond with poly A (Cerutti *et al.*, 1966), so its function in tRNA may be to break up the Watson−Crick pairs or to form alternative hydrogen-bonding structures. In the pre-RNA world, where alternative backbones to the ribose phosphate of RNA may have occurred, the strength of the DHU−A hydrogen bond may have been sufficient for double-helix formation. This might be possible in peptide nucleic acids (Nielsen *et al.*, 1991) where the double helix is not destabilized by the negative charges on the phosphates. Another role of dihydrouridine in the pre-RNA world may have been to alter the stability of a double helix by opening the DHU to ureidopropionic acid (UPA) thereby forming a negative charge on the strand. We therefore investigated the rate and equilibrium for ring opening of DHU, dihydrothymine (DHT), and 1-*N*-methyldihydrouracil (MDHU).

EXPERIMENTAL PROCEDURES

DHU and DHT were purchased from Calbiochem, and DHUR was purchased from Sigma. MDHU was prepared by acid-catalyzed ring closure of *N*-methyl-*β*-ureidopropionic acid, which was prepared by the reaction of potassium cyanate and N-methyl-*β*-alanine (Stark *et al.*, 1960; Stark, 1965). The *β*-ureidopropionic acid riboside (UPAR) was formed by overnight alkaline hydrolysis (0.1 M NaOH) of DHUR at room temperature (Cohn & Doherty, 1956). The HCl, phosphate, borate, carbonate, and NaOH buffers were measured with a Corning 250 pH meter, and the pH's were corrected for the temperature change based on $\Delta pH = \Delta pK_a$ with the ΔpK_a values from Robinson and Stokes (1959). Borate was only used as a buffer in experiments that did not contain a riboside since it is known to complex with sugars.

The rate as a function of phosphate buffer concentration was investigated for the ring opening of DHUR at pH 7.25 and 100 °C. The rate is given by the equation rate $= 2.25 \times 10^{-5}\ s^{-1} + 3.45 \times 10^{-5}(\Sigma PO_4)/[0.015 + (\Sigma PO_4)]$, where (ΣPO_4) is the total phosphate concentration in the buffer. At 0.02 M phosphate the rate is about 88% greater than the zero-buffer rate. For the ring-opening experiments with DHU, DHT, and MDHU, 0.02 M buffers were used (0.01 M for DHUR) as a compromise between control of pH and obtaining the zero-buffer rate. The solutions were heated in a Fisher 150 Temp heating block at the desired temperature. The DHU, DHT, and MDHU concentrations as a function of time were measured in pH 0 at 220 nm on a Hewlett-Packard 8452A diode array spectrophotometer after

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[1] Abbreviations: DHU, dihydrouracil; UPA, *β*-ureidopropionic acid; DHT, dihydrothymine; UIBA, *β*-ureidoisobutyric acid; MDHU, 1-*N*-methyldihydrouracil; MUPA, *N*-methyl-*β*-ureidopropionic acid; DHUR, dihydrouridine; UPAR, *β*-ureidopropionic acid riboside.

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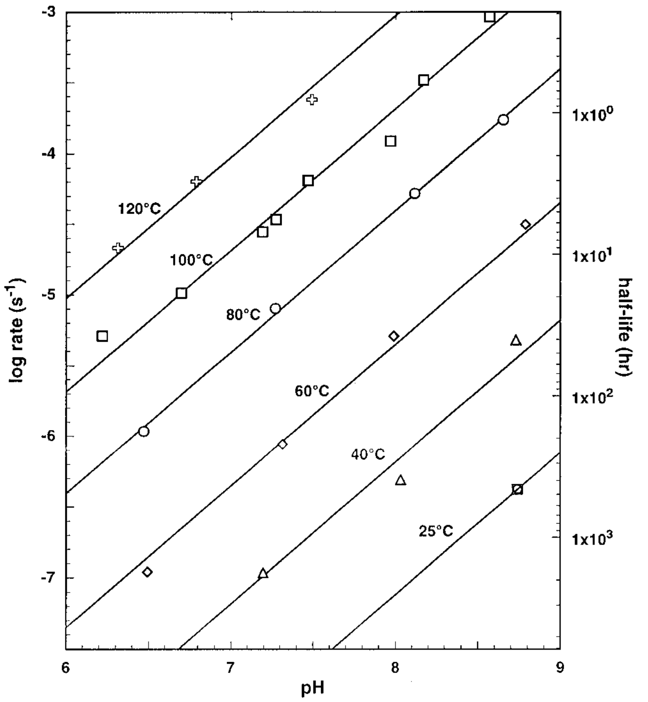


FIGURE 1: Ring-opening hydrolysis of dihydrouridine. The lines are fitted assuming a slope of 1 [i.e., rate = $k(OH^-)$]. The pH values of the various buffers have been corrected to the temperature of the reaction.

they were diluted 1:1 with 2 M HCl. The DHUR concentration and the concentration of the hydrolysis products were measured with a Beckman HPLC using an Alltech HEMA-IEC BIO 1000 Q 10U anion exchange column with a Kratos Spectroflow 757 absorbance detector at 220 nm. The eluant was an isocratic buffer of 0.001 M NaOH with a flow rate of 1.25 mL/min.

RESULTS

Ring-Opening Reactions. The pH rate profile for the ring opening of DHUR is shown in Figure 1 between 40 and 120 °C with one point at 25 °C. The rate is proportional to (OH^-) in this pH range. The Arrhenius plot of the ring opening of dihydrouridine at pH 7 is shown in Figure 2. The rate constant at pH 7 is given by

$$\log k\ (\mathrm{s}^{-1}) = 8.75 - 5010/T$$

The half-lives of DHUR at pH 7 are 9.1 h at 100 °C and 2.5 years at 25 °C. The heat of activation is 22.9 kcal/mol.

The pseudo-first-order rate constants as a function of pH for ring opening of DHU and several methylated derivatives are shown in Figure 3. The ring-opening hydrolysis of DHU and its substituted derivatives proceeds at a rate proportional to OH^- concentration in the pH range 6−9. The rate of hydrolysis of DHT ($k_{\mathrm{open}} = 3.14 \times 10^{-6}$ s^{-1}, 100 °C, pH 7) is 1.9 times slower than DHU ($k_{\mathrm{open}} = 6.01 \times 10^{-6}$ s^{-1}, 100 °C, pH 7). MDHU ring opening ($k_{\mathrm{open}} = 9.7 \times 10^{-7}$ s^{-1}, 100 °C, pH 7) is 6.2 times slower than DHU. DHUR ring opening is 3.5 times faster than dihydrouracil.

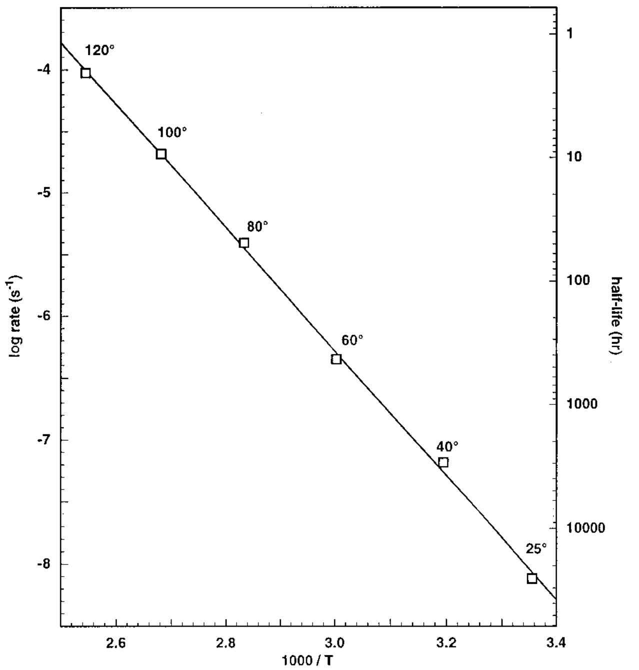


FIGURE 2: Arrhenius plot for the rate of ring opening of dihydrouridine at pH 7.

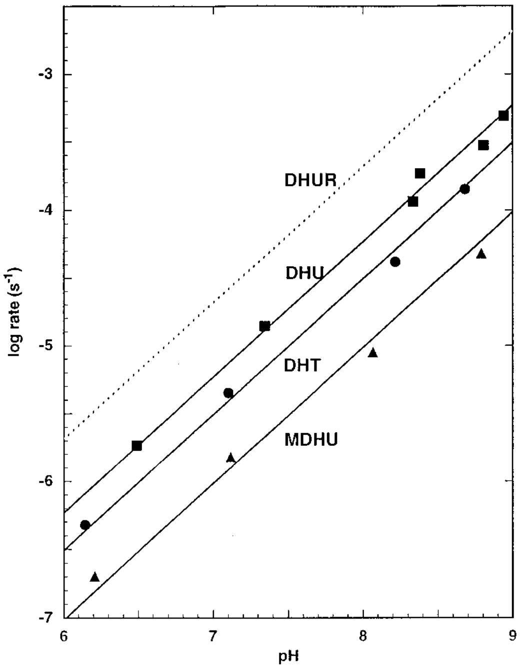


FIGURE 3: Rate of ring opening of various dihydrouracils at 100 °C. The points for DHUR (dotted line) are shown in Figure 2. The lines are fitted with an assumed slope of 1. The pH values of the buffers have been corrected to 100 °C.

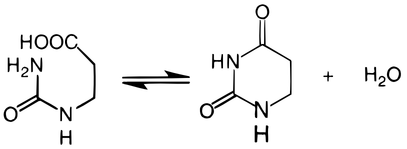
The rate of ring opening for DHU at pH 8 was also measured at 120, 80, and 60 °C. The Arrhenius curve for pH 8 [$\log k\ (\mathrm{s}^{-1}) = 8.91 - 4941/T$] extrapolates to $k = 2.18 \times 10^{-8}$ s^{-1} at 25 °C, in fair agreement with the value extrapolated to pH 8 from Sander's data (1969) at higher pH's of 1.70×10^{-8} s^{-1}. The ΔH^* is 22.6 kcal mol^{-1}, which is close to the 22.9 kcal mol^{-1} of DHUR.

Ring-Closing Reaction. The rates of ring closing in acid for UPA and UIBA have been measured by Pojarlieff and co-workers at several temperatures (Pojarlieff, 1967; Blagoeva, *et al.*, 1979). Extrapolating to 100 °C gives $k_{\mathrm{close}} = 1.40 \times 10^{-3}$ M^{-1} s^{-1} for UPA and $k_{\mathrm{close}} = 1.99 \times 10^{-3}$ M^{-1} s^{-1} for UIBA. The value for MUPA is 7.68×10^{-2} M^{-1} s^{-1}. Our measured value in 0.1 M HCl is close to this (8.5×10^{-2} M^{-1} s^{-1}). The rate of ring closure for UPAR was measured in 0.1 M HCl at 100 °C by measuring the appearance of DHUR with HPLC. The rate constant is 1.5

$\times\ 10^{-3}\ s^{-1}$. The loss of ribose from UPAR during this reaction was approximately 35%.

Equilibrium Constants for Ring Closure. The equilibrium constants for ring closure of UPA and UIBA in acid are given by Pojarlieff and co-workers (Pojarlieff, 1967; Blagoeva *et al.*, 1979) as $K_{eq}^{UPA} = 5.0$ and $K_{eq}^{UIBA} = 17.5$ at 60 °C. The value of K_{eq}^{UPA} is essentially independent of temperature. The values for UIBA and MUPA will also be taken as independent of temperature. Our attempts to measure the ring closure equilibrium for MDHU in acid were unsuccessful because of the nearly quantitative formation of MDHU. In the case of DHUR, the ribose was hydrolyzed off before equilibrium was reached. We therefore measured these equilibrium at pH's between 7 and 10.

The equilibrium reaction for closure of UPA to DHU in acid can be written as



The reaction is drawn over to the left at pH values greater than the pK_a by the ionization of the carboxyl of the UPA. At higher pH values, there is also ionization of the DHU with its p$K_a^{DHU} = 11.74$ (Koedjikov *et al.*, 1984). Let

$$K_{eq} = \frac{(\mathrm{DHU})}{(\mathrm{UPA})},\quad K_a^{\mathrm{UPA}} = \frac{(\mathrm{H}^+)(\mathrm{UPA}^-)}{(\mathrm{UPA})},\quad \text{and}$$

$$K_a^{\mathrm{DHU}} = \frac{(\mathrm{H}^+)(\mathrm{DHU}^-)}{(\mathrm{DHU})}$$

The apparent equilibrium constant as a function of pH is given by

$$K_{app} = \frac{\sum \mathrm{DHU}}{\sum \mathrm{UPA}} = K_{eq}\frac{(\mathrm{H}^+) + K_a^{\mathrm{DHU}}}{(\mathrm{H}^+) + K_a^{\mathrm{UPA}}}$$

where $\sum\mathrm{DHU} = \mathrm{DHU} + \mathrm{DHU}^-$ and $\sum\mathrm{UPA} = \mathrm{UPA} + \mathrm{UPA}^-$. In the pH region between 0 and 10 the equation is

$$K_{app} = \frac{K_{eq}}{1 + K_a^{\mathrm{UPA}}/(\mathrm{H}^+)}$$

The pK_a values for UPA and UIBA are given by Robinson and Stokes (1959). These are lowered by 0.05 pK unit to correct for the ionic strength of 0.05 M, giving values for UPA of 4.44 at 25 °C, 4.47 at 60 °C, and 4.60 at 100 °C. The values for UIBA are 4.41 at 25 °C, 4.44 at 60 °C, and 4.56 at 100 °C. We measured the pK_a of MUPA as 4.31 at 25 °C and ionic strength of 0.05 M by pH titration. By assuming the same temperature coefficient for MUPA as for UPA, we have p$K_a = 4.34$ at 60 °C and p$K_a = 4.47$ at 100 °C. The pK_a value for UPAR is taken as the same as UPA.

An equilibrium constant for ring closure of $K_{app} = 0.0137$ for MUPA was estimated from the rates of ring closing ($k_{close} = 9.9 \times 10^{-9}\ s^{-1}$) and ring opening ($k_{open} = 7.2 \times 10^{-7}\ s^{-1}$) at pH 8.8 and 60 °C. This gives $K_{eq} = 395$ by using p$K_a^{MUPA} = 4.34$. The equilibrium constant for ring closure of UPAR was obtained from the ratio of the rates of ring opening ($k_{open} = 4.0 \times 10^{-5}\ s^{-1}$ at pH 7.25 and 100 °C) and ring closure ($k_{close} = 1.65 \times 10^{-5}\ s^{-1}$ at pH 7.25 and 100 °C), giving $K_{app} = 0.413$. A similar experiment at pH 8.1 gave $K_{app} = 0.0690$. Using a pK_a of 4.60 for UPAR gives K_{eq} values of 184 and 218, respectively, or an average of $K_{eq} = 201$.

pH−Rate Profile for Ring Opening and Ring Closing. With the equilibrium constants for ring closure combined with the rate of ring opening in base and ring closure in acid, it is possible to construct the pH−rate profile for both reactions. Since the ratio of the rates is the apparent equilibrium constant at each pH, we have for the acid-catalyzed part of the curve

$$k_{open} = k_{close}/K_{eq}$$

For the basic part of the curve, we have

$$k_{close} = k_{open}K_{eq} = \frac{k_{open}K_{eq}}{1 + K_a/(\mathrm{H}^+)}$$

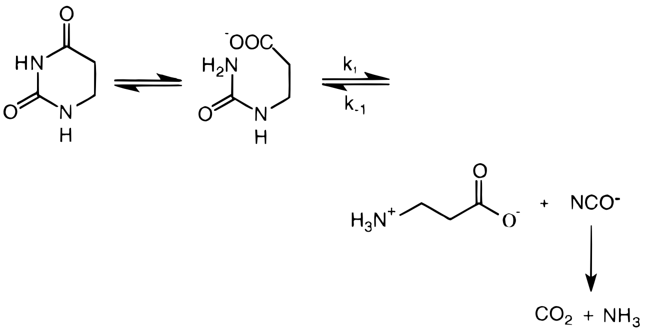
The curves are shown in Figure 4. It can be seen that the rate of ring closing in acid is in the same sequential order as the equilibrium constant. The same holds for the base-catalyzed ring closure except that DHUR is considerably faster than expected on the basis of the K_{eq}.

In the case of ring opening in acid, the rates are not much different, again except for DHUR. Thus, in the acid-catalyzed reaction much of the difference in K_{eq} resides in k_{close} rather than in k_{open}. In basic solution, the rates of ring opening are in the inverse order of K_{eq} except for DHUR. In base the differences in K_{eq} are shared more evenly between k_{open} and k_{close}.

The effect of *gem*-methyl groups on the stability of rings and their rates of ring closure (Thorpe−Ingold effect) has been recognized (Kirby, 1980). The data for DHU and DHT are in accord with this, but the effect with MDHU is particularly large. This has been previously noted for the acid-catalyzed ring closure of MUPA (Blagoeva *et al.*, 1979).

It is not clear why DHUR/UPAR is anomalous. The hydroxyls on the ribose may be acting as internal general acids or bases or the inductive effect of substituents on the nitrogen could be particularly strong.

Decomposition of Ureidopropionic Acid. Ureidopropionic acid decomposes to cyanate and *β*-alanine in a reversible reaction, with the cyanate decomposing to CO_2 and NH_3 in an irreversible reaction (Hagel *et al* , 1971).



A value of $k_{-1} = 9.4 \times 10^{-5}\ M^{-1}\ s^{-1}$ at 30 °C was measured by Stark (1965), which is adjusted to $k_{-1} = 4.9 \times 10^{-5}\ M^{-1}\ s^{-1}$ for 25 °C using the heat of activation for urea synthesis of 23.2 kcal mol^{-1}.

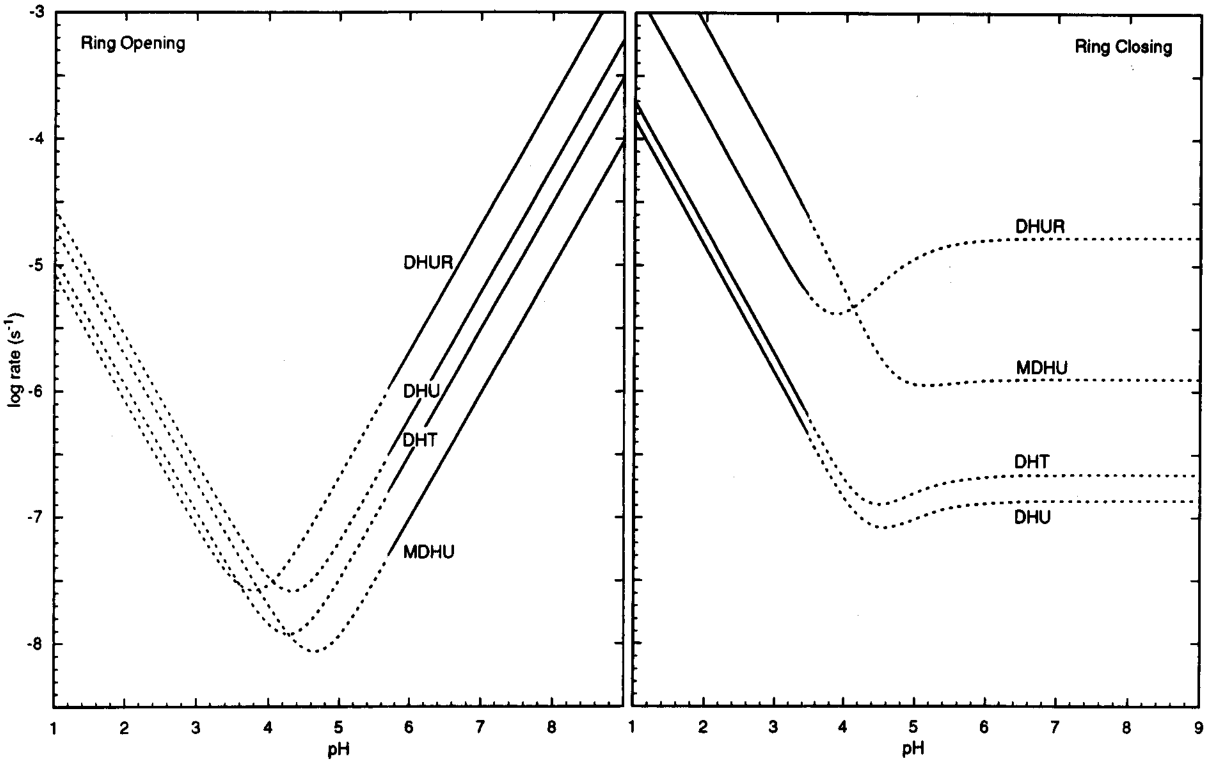
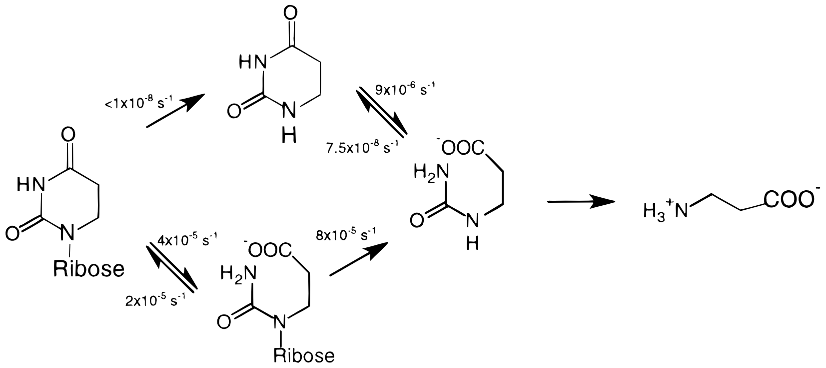
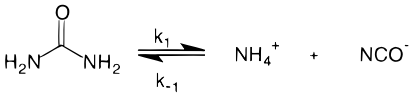


FIGURE 4: pH−rate profile of ring opening and ring closure of several dihydropyrimidines. The solid lines are based on experimental measurements (extrapolated in the case of ring closure), and the dotted lines are based on $k_{open} = k_{close}/K_{eq}$ and $k_{close} = K_{eq}k_{open}$.

Scheme 1. Approximate Rates for the Decomposition Pathways of DHUR at pH 7.25





$$H_2NCONH_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} NH_4^+ + NCO^-$$

Estimating $k_1 = 1.79 \times 10^{-10}$ s^{-1} at 25 °C from the value for methylurea (Shaw & Grushkin, 1960) gives $K_{eq} = k_1/k_{-1} = 3.65 \times 10^{-6}$. This can be compared to the 25 °C values for urea (Frost & Pearson, 1961) of $k_1 = 5.40 \times 10^{-10}$ s^{-1} and $k_{-1} = 3.94 \times 10^{-5}$ M^{-1} s^{-1}, giving $K_{eq} = k_1/k_{-1} = 1.37 \times 10^{-5}$. Therefore the K_{eq} for UPA dissociation is approximately a factor of 3 less favorable than urea.

Decomposition of Dihydrouridine and UPA Riboside. The scheme for the decomposition of DHUR is more complex than that for UPA since there is loss of ribose in addition to ring opening (Scheme 1). Not shown is the loss of cyanate from UPAR giving β-alanine riboside. We found no evidence for this reaction, but it would have been difficult to see it with our HPLC scheme.

In basic solution the reaction of DHUR is almost entirely ring opening to UPAR. In acid solution the ribose is hydrolyzed off. The rate equation is taken to be

$$\text{rate} = k(\text{DHUR})(\text{H}^+)$$

with a rate constant of 8.3×10^{-3} M^{-1} s^{-1} at 100 °C based on a measurement with 0.1 M HCl. We could detect no loss of ribose from DHUR at pH 7.25 and 100 °C ($k < 10^{-8}$ s^{-1}). The acid-catalyzed rate extrapolated to pH 7.25 is $k = 4.7 \times 10^{-10}$ s^{-1}, which is consistent with our inability to detect the loss of ribose from DHUR. There is considerable loss of ribose from UPAR in neutral solution, and presumably in acid, but this was not determined because of the competing reaction of ring closure.

DISCUSSION

In Vivo Stability of Dihydrouridine. The results on the rate of ring opening of DHUR shows that this nucleotide should be stable for organisms growing at temperatures below about 80 °C ($t_{1/2}$ = 2.2 days). The 80 °C is very approximate because it is not known what level of DHUR degradation is tolerable. The half-life at 37 °C is 203 days at pH 7 (81 days at pH 7.4), which suggests that DHUR should be stable in mammals to nonenzymatic ring opening and loss of ribose. DHUR has been found in the urine of rats and is excreted quantitatively (Topp *et al.*, 1993). This suggests that there is no enzymatic degradation of DHUR.

At temperatures above 100 °C the rate of ring opening ($t_{1/2}$ = 9.1 h) is comparable to the doubling time of the hyperthermophiles (~7 h), and the problem becomes acute at 110 °C (4.1 h). Ring opening of the DHUR would be followed by loss of the UPA and breaking of the ribose phosphate chain. The data shown in Scheme 1 indicate that DHUR does not lose DHU before opening the ring to UPAR.

The half-life of ring opening of 9 h at 100 °C is not that rapid compared to the optimum doubling time of hyperthermophiles, which is 1−3 h (Pley *et al.*, 1991; Kurr *et al.*, 1991; Völkl *et al.*, 1993). However, the doubling times increase steeply on both sides of optimum temperature, and under natural growth conditions the loss of DHUR is likely to be rapid compared to the doubling time of the hyperthermophiles. Therefore, it is clear that tRNA molecules containing DHUR are unsuitable for hyperthermophiles unless there is some mechanism to stabilize the DHUR in the structure. The stabilization could come from hydrogen bonding or hydrophobic bonding in the tRNA structure. The loss of tRNA molecules could be overcome by resynthesis, but this would entail considerable energy consumption. Thus the absence of DHUR in the hyperthermophiles is understandable.

Although DHUR is extremely rare in Archaea, there are a few exceptions such as *Thermoplasma acidophilum* and *Methanobacterium thermoautotrophicum* that grow around 60 °C (Edmonds *et al.*, 1991). In addition, there is *Thermococcus* sp. that grows at 98 °C (Edmonds *et al.*, 1991) and a Eubacterium *Thermus thermophilus* growing between 48 and 85 °C which contains DHUR (Yokoyama *et al.*, 1987). It is clear that the DHUR in these organisms must have limited stability, and the tRNA's need resynthesis unless there is a stabilization mechanism in the tRNA structure.

It is not clear whether DHUR is absent from most mesophilic Archaea, some of which grow in Antarctic waters (DeLong *et al.*, 1994), and these tRNA's should be examined. If DHUR is prevalent in mesophilic Archaea, the absence in hyperthermophiles could be explained by thermal instability. If low-temperature Archaea descended from hyperthermophiles, then DHUR became incorporated in the tRNA of the mesophiles because of some selective advantage, but this may not happen in every case.

Possible Prebiotic Roles of Dihydrouridine. β-Alanine, β-aminoisobutyric acid, and other β-amino acids are well established prebiotic amino acids (Wolman *et al.*, 1972) and are also found in the Murchison meteorite (Kvenvolden *et al.*, 1971; Peltzer *et al.*, 1984). Small amounts of hydantoic acids and hydantoins have also been found in the Murchison meteorite (Cooper & Cronin, 1995). It is likely that β-ureido acids and dihydrouracils are also present, but they were not looked for.

The presence in the primitive oceans of β-alanine and other β-amino acids together with urea would result in the formation of the UPA and other β-ureido acids. DHU, DHT, and other dihydropyrimidines would also be present at equilibrium with the DHU/UPA ratio depending on the pH.

It is clear that DHUR is an unlikely compound for use in the earliest genetic material because of the instability of its glycosidic bond and also because ribose is an unlikely prebiotic compound (Larralde *et al.*, 1995). However, DHU attached to a backbone different from ribose phosphate is an attractive possibility. The hydrogen bonding to adenine may be stronger on a different backbone, and other structural roles are possible, such as breaking up the Watson−Crick structures or the ability to form special structures in early ribozymes.

The marginal stability of the ring of any DHU's in the pre-RNA world may have been an advantage. There is always a problem of separating a double strand of genetic material. One way around the problem is to vary the pH. At high pH (e.g., pH ≈ 8.5) DHU rings would open, and the negative charges would push the chains apart. If the separated strands were then brought to more acidic pH's, the UPA would close to DHU, removing the negative charge and allowing the double strands to reform. Carboxylic acid groups on the pre-RNA bases (e.g., orotic acid or uracil-5-acetic acid) could also do this, as could amines or other bases on the uracil, but DHU would have an hysteresis effect that might be desirable.

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