Article

The effects of high pressure and pH on the hydrolysis of cytosine at high temperatures

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Abstract

There has been much speculation regarding the environment in which life originated but it has still to be determined what environmental chemical and physical conditions were necessary for the evolution of self-replicating chemical systems. Opinion is strongly divided on whether life arose on earth under hot or cold conditions; the hot-start and cold-start scenarios respectively. While it has been determined that DNA, RNA and their components are chemically unstable at high temperatures, there have yet to be studies into the role of high pressures. We report experimental results showing that high pressures *increase* the rate of hydrolysis of cytosine to uracil. The results have been obtained with a specialised high-pressure NMR cell. These results provide evidence of a low pressure/low temperature environment being necessary in the origin of life, at least for RNA-based life forms.

Introduction

There are many, varied and hotly debated scenarios for the origin of life on earth. Most scenarios have RNA-based life forms preceding the extant protein-based life forms but this is also vigorously disputed.[1] There are hot-start[2] *versus* warm-start[3] *versus* cold-start[4] scenarios with hot-start scenarios popularistically featuring the energy- and chemically-rich deep ocean black smokers where hyperthermophiles from the kingdoms Archaea and Crenarchaea, often taken as the closest living descendants of the first protein-based life forms,[5] thrive at temperatures between 80°C and 110°C and at high hydrostatic pressure. However, it is well known that at least at ambient pressure of one atmosphere (0.1 MPa), folded RNA structures denature at temperatures less than 70°C *in vitro*.[6] A pioneering study on the chemical stability of RNA components by Levy and Miller[7] found that the nucleobase cytosine hydrolysed to uracil (Fig. 1a) with a half-life of just 19 days at 100°C and ambient pressure, compared to 340 years at 25°C. This rate of hydrolysis is at least 15 times faster than the rates of decomposition of other nucleobases (guanine, 0.8 year and adenine, 1 year at 100°C). Assuming that earliest life forms were rather inefficient and not adapted and stripped down for speedy turnover, in the manner of many extant Prokarya, Crenarchaea and Archaea, these seminal results rendered unlikely a hot-start scenario for RNA-based life forms.

However, these studies did not address the effect of pressure, and whether or not high pressure countered or exacerbated the deleterious effects of high temperatures on the physical and chemical stability of RNA. It is believed that high pressure may have had an important role in the development of life on earth.[8] We report here a comprehensive study on the effects of high hydrostatic pressure on the chemical stability of cytosine and cytidine at 100°C along with brief studies on the effects of pH on chemical stability under high pressure. We show that the rate of hydrolysis of cytosine and cytidine at 100°C increases with increasing pressure and, further, that this rate is minimised for cytosine at pH ~7.

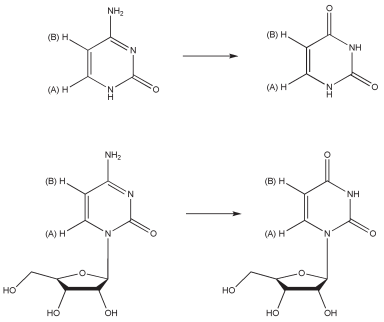

Fig. 1. The hydrolysis of (a) cytosine and (b) cytidine to uracil and uridine respectively. The atom labelling scheme is shown for H(A) and H(B).

This increase in the rate of hydrolysis of cytosine at high pressure may lead to the rethinking of high temperature/high pressure origin of life theories which involve cytosine in genetic coding.

Materials and methods

High pressure NMR apparatus

The commercially available on-line high pressure NMR cell was purchased from Daedalus Innovations. The high pressure cell consists of a zirconia tube (inner diameter 3 mm, outer diameter 5 mm) attached to an aluminium manifold, connected to a long stainless steel tube, connected to a remote hand pump. Pressure is applied to the aqueous sample in the NMR cell *via* an immiscible hydraulic fluid (paraffin oil). This setup allows the pressure on the sample to be set at any value between 0.1 and 250 MPa (measured with a HiP Bourdon Gauge). A specially designed rig allowed the NMR cell to be reproducibly positioned in the spectrometer and to be safely moved under pressure from an external oil bath at temperatures up to 373 K to the spectrometer, allowing other users to access the instrument over the time course of experiments. The cell was interfaced

with a Bruker Avance 500 MHz NMR spectrometer. The sample was positioned in a commercial 5 mm ^{1}H-detection inverse probe with an *x, y, z*-field gradient coil.

Samples

A stock solution of cytosine or cytidine (Sigma), 3-(trimethylsilyl)-2,2',3,3'-tetradeuteropropionic acid (TMSP-d4) (Merck) and sodium azide was added to a phosphate buffer in H_2O/D_2O (90:10 v/v) to give a final solution of 1 mM cytosine, 0.05 M phosphate buffer, 0.02 mM sodium azide and 0.2 mM TMSP-d4. The ionic strength of all solutions was adjusted to 0.2 M with NaCl. The TMSP was added to act both as an NMR reference and as an internal concentration reference. Due to the effect of pressure on pH in a phosphate buffer, the pH values at atmospheric pressure for the high pressure samples were corrected to give the true pH at 298 K while under pressure.[9] Table 1 details the incubation conditions of the individual samples.

Hydrolysis measurements

Samples were inserted into the high pressure cell and brought up to pressure. A ^{1}H NMR spectrum was recorded and the sample was then incubated in an oil bath at 373 K for a period of at least 135 hours while maintaining the pressure at the target value (no leaks were observed over periods of weeks). During this time further ^{1}H NMR spectra were recorded periodically at 298 K after which the sample was promptly restored to 373 K. All NMR spectra were recorded with 64 scans and a relaxation time of 10 s with a presaturation pulse to suppress the water peak. The integrals of the NMR peaks corresponding to protons H(A) and H(B) for both cytosine and uracil (Fig. 1a) were measured for each spectrum using the TMSP peak as a reference. From this, a plot of ln concentration *versus* time was used to determine the rate constant for hydrolysis, *k*, for each sample.

Results

The hydrolysis of cytosine was monitored at 373 K and at pressures of 0.10, 50, 100, 150 and 200 MPa (Fig. 2a). The corresponding data for hydrolysis of cytidine at 373 K at pressures of 0.1 and 150 MPa are shown in Fig. 2b. The processes are first order in all cases, as all plots of ln [cytosine] *versus* time are linear. The NMR spectra, especially of cytidine, were closely inspected for evidence of decomposition additional to the deamination to uracil. No evidence was found by way of peaks not assignable to uracil and cytosine (or cytidine). Moreover, the integrals of the H(A) and H(B) proton signals for uracil and cytosine (or cytidine), normalised to those of TMSP, were constant within 4.0% over the time course of the experiment.

The rate constants and associated estimated standard deviations derived as the slope of these plots are tabulated in Table 2, alongside the half-lives, for cytosine (or cytidine). The half-life decreases monotonically as the pressure increases from 0.1 to 200 MPa, from 19.3 ± 1.6 days at 0.10 MPa to 9.1± 0.4 days at 200 MPa. The half-lives for cytidine at 0.10 MPa and 150 MPa and 373 K are 1.89 ± 1.6 days and 9.3 ± 0.8 days, respectively (no measurements taken at 200 MPa). The half-lives for hydrolysis of cytidine at both low and high pressures are shorter than those for cytosine, which lacks the ribose group attached to the nucleobase.

Table 1. Pressure and pH incubation conditions for individual samples including corrected pH values at 0.1 MPa

	Pressure / MPa	pH under pressure	pH at 0.1 MPa
Cytosine	0.1	6.00	6.00
	0.1	7.00	7.00
	0.1	8.00	8.00
	50	7.00	7.20
	100	7.00	7.39
	150	6.00	6.56
	150	7.00	7.56
	150	8.00	8.56
	200	7.00	7.71
Cytidine	0.1	7.00	7.00
	150	7.00	7.56

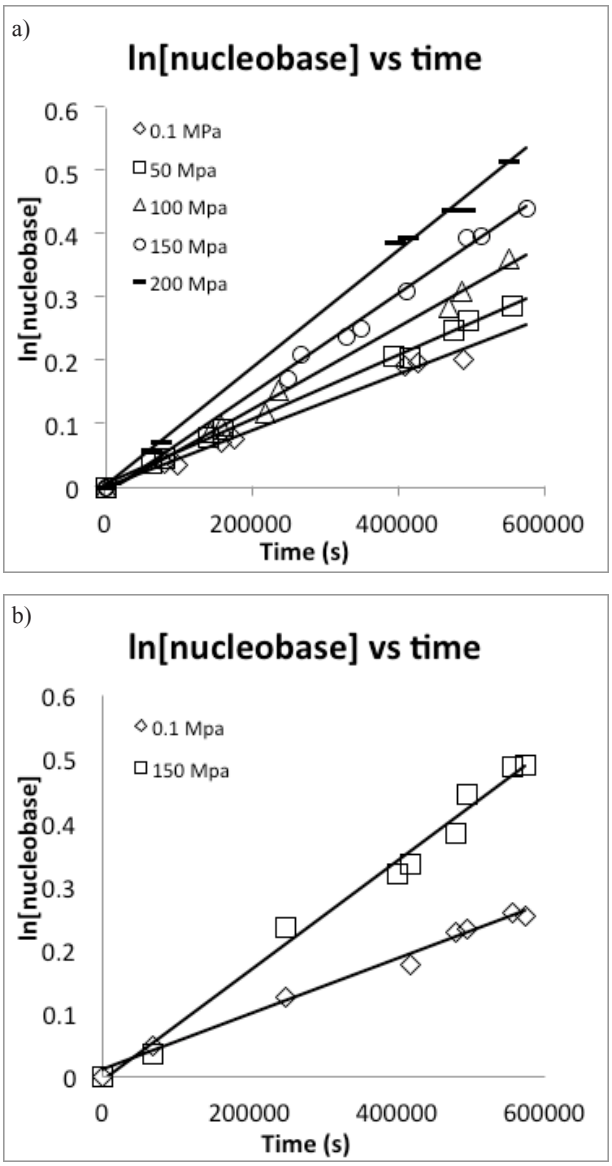

Fig. 2. Plot of ln [nucleobase] *vs* time at 373 K for (a) cytosine at various pressures and (b) cytidine at 0.10 MPa and 150 MPa. The rate constant for hydrolysis of cytosine to uracil is given by the slope of the fitted line

Table 2. The rate constants and associated estimated standard deviations for each incubation condition from Table 1. Half-lives are displayed below each rate constant in square brackets.

	Pressure (MPa)				
	0.1	50	100	150	200
	k / s^{-1} x10^{-6} [$t_{1/2}$ (/day)]	k / s^{-1} x10^{-6} [$t_{1/2}$ (/day)]	k / s^{-1} x10^{-6} [$t_{1/2}$ (/day)]	k / s^{-1} x10^{-6} [$t_{1/2}$ (/day)]	k / s^{-1} x10^{-6} [$t_{1/2}$ (/day)]
Cytosine pH 6.0	0.59 ± 0.07 [13.55]			0.87 ± 0.07 [9.261]	
Cytosine pH 7.0	0.42 ± 0.04 [19.29]	0.49 ± 0.04 [16.45]	0.62 ± 0.03 [13.04]	0.72 ± 0.06 [11.13]	0.88 ± 0.04 [9.141]
Cytosine pH 8.0	0.42 ± 0.03 [18.91]			0.78 ± 0.04 [10.34]	
Cytidine pH 7.0	0.44 ± 0.04 [18.22]			0.87 ± 0.08 [9.254]	

The pressure profile of the rate constants for cytosine at 373 K is shown in Fig. 3, as a plot of ln k *vs.* pressure p,

$$\frac{\partial lnk}{\partial p} = -\frac{\Delta V^{\ddagger}}{RT} \qquad \text{Equation 1}$$

where R is the gas or universal constant and T is the absolute temperature in K. From this the value of the reaction volume of activation, $\Delta V^{\ddagger}$, was calculated to be: for cytosine -11.7 ± 1.2 cm^3 mol^{-1}, and for cytidine, -14.6 cm^3 mol^{-1}. These values are similar in magnitude to the molar volume of water, 18.01 cm^3 mol^{-1}.

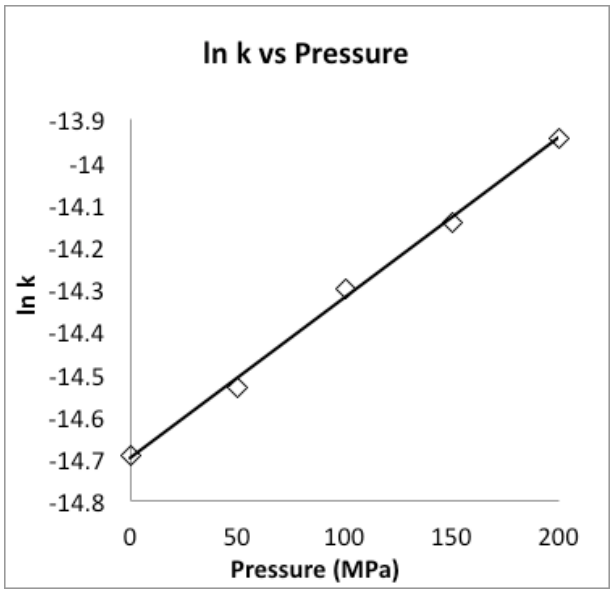

Fig. 3. Plot of the log of the rate constant k for the hydrolysis of cytosine *vs.* pressure p. From the slope of the line $-\Delta V^{\ddagger}/RT$ the activation volume $\Delta V^{\ddagger}$ can be derived.

A brief examination of the pH dependence of cytosine hydrolysis was carried out at pressures of 0.1 and 150 MPa (Fig. 4). It is seen in both curves that the rate of hydrolysis is the slowest in the region of pH 7. Initial measurements made at pH 4.00 and 373 K (data not shown) were consistent with this, as the rates calculated were much larger than those at pH 7.

Discussion

The relatively fast rate of hydrolysis of cytosine to uracil at

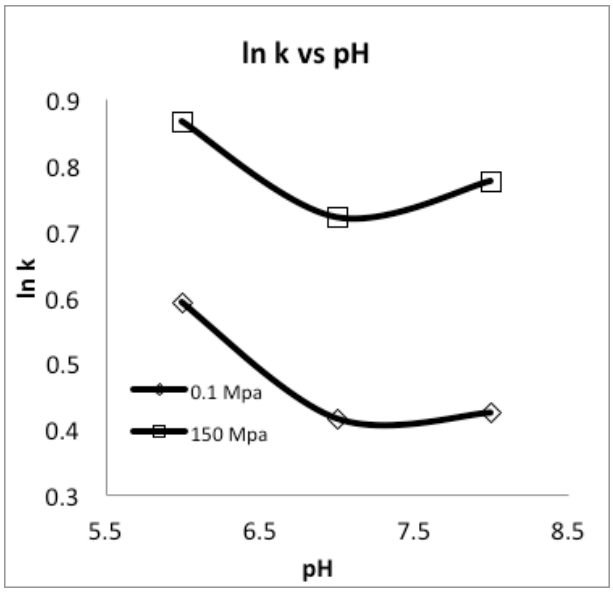

Fig. 4. The pH profile for the hydrolysis of cytosine at 0.1 MPa and 150 MPa. pH values shown at 150 MPa are those corrected for pressure effects. It can be seen that there is a minimum in the rate of hydrolysis just above pH 7. The lines are for visual reference only.

100°C and ambient pressure, compared to geological time scales, has previously been cited as a limiting factor for the evolution of life at high temperatures.[7] With regard to all the possible locations for the development of life, there is a wide range of different conditions to consider, from acidic high pressure, high temperature deep sea black smokers to alkaline low pressure, moderate temperature saline springs. Because of this, we have examined the effect of pressure and pH on cytosine hydrolysis to determine whether there is any possibility of high pressures offsetting the negative effects of high temperatures on nucleotide chemical stability.

Somewhat counter to our expectations, the rate of hydrolysis of cytosine increases with pressure such that at 200 MPa the rate constant for deamination is doubled compared to that measured at an ambient pressure of 0.10 MPa. The half-life determined at 0.10 MPa and 100°C in our measurements of 19.3 ± 1.6 days compares well with that of 19 days determined by Levy and Miller[7] at 0.10 MPa 100°C

for cytosine at pH 7 under the same conditions (0.05 M phosphate buffer at an ionic strength 0.2 M). We observed that under the same conditions, the rate of hydrolysis of cytidine is faster than that of cytosine, the difference being more pronounced and significant at higher pressures. Respectively, the half-lives for hydrolysis at 100°C are; for cytosine, at 0.10 MPa, 18.9 ± 1.6 days and 19.3 ± 1.6 days, and for cytidine, at 150 MPa, 9.3 ± 0.8 days and 11.1 ± 0.9 days. This shows differences of 0.4 days to 1.8 days. Similar differences were observed for results obtained at pH 4.8 (data not shown).

From the plot of ln *k* vs p, the activation volume, $\Delta V^{\ddagger}$, for the hydrolysis of cytosine and cytidine at 100°C was calculated to be -11.7 ± 1.2 $cm^3\ mol^{-1}$ and -14.6 ± 1.4 $cm^3\ mol^{-1}$. Respectively, these results appear to be typical for biochemical processes, where the magnitude of values for $\Delta V^{\ddagger}$ are within the range of 0 to 50 $cm^3\ mol^{-1}$.[10] Our negative value corresponds to an increased rate of deamination with increasing pressure. The difference between the two results for cytosine and cytidine indicate that the hydrolysis rate for cytidine, which has the attached ribose ring, is slightly more susceptible to the effects of pressure.

Compared to the half-life at 0.1 MPa (atmospheric pressure) of 19.3 days, the value of the half-life, calculated via equation 1, is 13% lower at 38.2 MPa, the pressure corresponding to the mean ocean depth of 3790 m.[11] At the deepest ocean pressure of 110 MPa, corresponding to a depth of 10920 m,[12] the half-life is 33% shorter than that at atmospheric pressure. Here, the result is significantly different but applies to only a small portion of deep sea environments.

The examination of the pH dependence of the hydrolysis of cytosine has shown that the pH dependence of hydrolysis at high pressures is similar to that at ambient pressure (Fig. 4). Both plots indicate that the pH that will result in the lowest rate of hydrolysis is located somewhere just above pH 7. At atmospheric pressure this result is to be expected since the nucleobases are the most stable at pH 7.[13]

The instability of biomolecules has long been recognised as a weakness in the argument for a hot-start origin of life theory.[14] Cytosine has been observed to be the least stable of all the nucleobases having a half-life of 340 year at 298 K (compared to ~ 10000 year for adenine and guanine at 298 K) but having a half-life of only 19 days at 373 K (compared to ~ 1 year for adenine and guanine). These half-lives are very short on the geological time scale and therefore decrease the likelihood of a high temperature origin of life involving cytosine. It had been argued that the instability of RNA and its components at high temperatures may be offset by high pressures in a high temperature/high pressure theory.[8] Our results show that this is not the case and that it is in fact the opposite for the hydrolysis of cytosine and cytidine. This is a solid argument against a high temperature/high pressure origin of life theory involving cytosine or derivatives. There is also a possibility for an origin of life theory that does not involve cytosine as a nucleobase, in genetic material that is either two base coded with just adenine (A) and uracil (U) or where the cytosine-guanine (CG) pair has been substituted for an alternate base pair (isoguanine and isocytosine, diaminopurine and U, diaminopyrimidine and xanthine).[7] However, numerical simulations indicate that a model containing only A and U does not lead to the unique stable folded RNA structures necessary for catalytic functions.[15,16] This would be an important limitation if proteins had yet to be used for catalytic function.

Conclusions

As previously indicated, the rate of hydrolysis of cytosine at 100°C is relatively short on the geological time scale. Our data on the chemical stability of cytosine and cytidine under high pressure conditions at 100°C indicates that both cytosine and cytidine have significantly faster rates of hydrolysis at high pressure. Data also show that there is a rough translation of the pH dependence on the rate of hydrolysis for cytosine at both 0.10 MPa and 150 MPa. These results favour a low temperature/low pressure origin of life theory over a high temperature/high pressure theory.

What still remains unknown is the effect that specific adjuvants, such as amino acids, short peptides, and magnesium ions may have on the chemical stability of cytosine and its derivatives while under pressure. There is also the question of the chemical stability of cytosine within folded RNA/DNA molecules under pressure. A study of the chemical and physical stability of RNA/DNA molecules is to be a major part of current work.

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