Far UV Photolysis of Uracil and Cytosine in Phosphate Solution

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Abstract The photolysis of nucleobases, nucleosides and nucleotides (NA) was enhanced by phosphate under irradiation of medium pressure mercury lamp (MPML). Uracil and Cytosine in phosphate solution were selected to study the mechanism of phosphate effect. Photoproducts were produced in the irradiated uracil and cytosine of phosphate solution which have been isolated by anion exchange resin. Ultraviolet irradiation (190-220nm) of uracil in 0.05 mol/dm³ phosphate buffered solution at pH 8-9 leads to the production of a novel compound C₄H₅N₂O₆P which has been identified by UV, ¹H-NMR spectroscopy and LC/MS/MS. The formation mechanism of the photoproduct and the kinetics were studied.

Keywords: UV photolysis, uracil, cytosine, phosphate

Introduction

The sun was cooler four billion years ago. Ultraviolet radiation most likely was strong despite a cooler sun because there was no ozone shield. Phosphorus, in the form of phosphate, is a conspicuous constituent of many important biomolecules, in particular nucleic acids. The Earth contains plenty of phosphates, but locked up in water-insoluble calcium phosphate in the mineral apatite. The identification of a suite of alkyl phosphonic acids in the Murchison meteorite has created a new perspective. It is probable that a significant proportion of the Earth primitive atmosphere is of exogenous origin (Chyba & Sagan, 1992). In the RNA world scenario (Gilbert, 1986) life began with the spontaneous assembly of a self-replicating RNA molecule. In 1995, Robertson and Miller reported that cyanoacetaldehyde was allowed to react with high concentrations (1-20 mol/dm³) of urea at 100°C to afford cytosine in good yield. Uracil would be formed by deamination of cytosine. However, the hydrolytic instability of cytosine and photolysis of nucleobases by far UV absorption of phosphates would appear to preclude the role in the origin of life. In this study, it was found that the photolysis of bases, nucleosides, nucleotides was sharply enhanced by phosphate under the irradiation of medium pressure mercury lamp (MPML). In order to explain this phenomenon, the photolytic system of uracil and cytosine in phosphate solution was selected and studied.

Experimental

Chemicals. All nucleobases, nucleosides and nucleotides were obtained from Sigma Chemical company. Amino acids were from Sigma and Shanghai Biochemistry Institute. Phosphate salts, K₂HPO₄·3H₂O were supplied by Beijing Chemical Reagent Factory. Triply distilled water was used to prepare all the solutions.

Determination of the radiation intensity of MPML under 200 nm. According to Halmann and Platzner's report (1966), when 5.0 mol/dm³ CH₃OH was irradiated under UV 200 nm, the quantum yield of hydrogen was equal to 0.5. We used the coefficient and the quantity of hydrogen which was produced under the irradiation of 5 mol/dm³ CH₃OH to calculate the radiation intensity of MPML under 200 nm. Hydrogen was analyzed by gas chromatography. In our experiment, the radiation intensity is measured in values of 3.916 × 10⁻⁶ Einstein cm⁻³ min⁻¹. The relative quantum yield (ϕ) was calculated by defining ϕ of the 5 × 10⁻⁵ mol/dm³ 5'-CMP in 0.25 mol/dm³ phosphate solution as unity.

Far UV irradiation. In our experiment, a 450W middle pressure mercury lamp (MPML) was employed as an irradiation source (Wang, KJ, 1995, 1997). The MPML emits a continuum spectrum possibly covering the range from less than 190 nm to visible light. Photolysis was done in a 1cm length quartz cell that was placed 20 cm away from MPML to keep the temperature of the solution in the cell at about 30°C. The irradiation time was controlled by an electric automatic shutter. The solution was contained 10⁻⁴ mol/dm³ nucleobase and 0.05 mol/dm³ K₂HPO₄. Deoxygenation was carried out by bubbling 99.99 % pure nitrogen through the solution stored in a special quartz tube (ϕ 1 × 5cm) for 30 min when necessary.

Preparation of product was performed in large scale. 200 ml solutions containing 10⁻⁴ mol/dm³ nucleobase and 0.05

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mol/dm³ K₂HPO₄ (pH 8.5) were irradiated for 30 minutes in a glass vessel surrounded by ice-water to keep the temperature < 5 °C. The solution was mixed by a magnetic stirrer.

Filter UV light experiment: In the emission spectra of MPML, the UV quanta less than 190nm can be filtered by 1 cm length water filter which is placed before the photolytic solutions. Similarly, the UV quanta less than 220 nm are filtered by 1 cm length of 1 mol/dm³ phosphate solution.

High Performance Liquid Chromatography (HPLC): The column was Zorbax SB C₁₈ column 4.6×250 mm. The sample (10 ml) was injected into the column and eluted under isocratic conditions with triply distilled water at a flow rate of 1 ml/min. The UV detector was set at 270 nm. The experiment was performed at 25°C.

LC/MS/MS: A HPLC Varian 9010 (USA) coupled with a Vestec Model 201 Mass spectrometer was used for the HPLC/MS analysis. The interface was of the ionspray type and the mass spectrometer operated in the positive-ion ionspray discharge ionization mode. Methanol was used as organic modifier. The ion source temperature was 250 °C and positive-ion spectra between 50 and 300 u were recorded at 3.2 sec per scan. HPLC conditions were the same as above.

The photolytic loss of NA: The phosphate effect on nucleobases is determined by the photolytic loss of NA. It is defined as follows:

$$\text{The photolytic loss of NA(\%)} = \frac{[\text{NA}]_{\text{unirradiated}} - [\text{NA}]_{\text{irradiated}}}{[\text{NA}]_{\text{unirradiated}}} \times 100$$

The concentration of NA is determined by the peak area of NA in HPLC.

Isolation of photoproducts: 200ml photolytic solution was concentrated to 50 ml, then the solution was chromatographed on an anion exchange column of Amberlite CG 400 II, 200-400 mesh, Cl form. The resin, after activation with 2.0 mol/dm³ alkali and 2.0 mol/dm³ acid and washing with water, has been converted to formate form with 2 mol/dm³ ammonium formate. After washing the excess NH₄⁺ with water, the resin maintained equilibrium with 0.1 mol/dm³ formic acid. Elution was carried out at the rate of 1.0 ml/min. The eluents were collected in each fraction of 12 ml. The column and the collected samples were maintained at room temperature throughout the chromatography. A sample of each fraction was analyzed by ultraviolet spectrometry immediately after it emerged from the column. The total eluent containing each fraction was combined, distilled to dryness in vacuum with a bath temperature of 50°C. The major photoproduct was concentrated, analyzed by UV, ¹H-NMR spectroscopy and LC/MS/MS.

Results

The enhancement of NA photolysis by phosphate

MPML emits a continuum spectrum covering the range from less than 190 nm to visible light, which can be used to imitate the Sun at the primitive period. The photolysis of nucleobases under MPML can be enhanced sharply by phosphate which exhibits a weak absorbance from 300 nm to 220 nm and a steep absorption edge below 200 nm (Halmann, *et al.*, 1965). The filter experiments demonstrated that the wavelength dependence of the enhancement is 190-220 nm in the spectrum of the MPML. The maximum enhancement effect of the phosphate on the photolysis of NA covers the range 0.05 ~ 0.1 mol/dm³ of phosphate concentration. Experimental results are given in Fig. 1.

The phosphate effect affected by photolysis time, concentrations of nucleobase, phosphate and pH. The photolysis loss of NA was increased when the photolysis time increased, as shown in Fig. 2. In order to inhibit the secondary reaction, the photolysis time was controlled to less than 15 minutes at which the photolytic loss was less than 60%. The loss was decreased with the increasing concentration of base (shown as Fig. 3) due to the competitive absorption of UV light between nucleobase and phosphate. In the present experiment, the concentration of nucleobase was set as 1.0×10⁻⁴ mol/dm³. When the concentration of phosphate was 0.05 ~ 0.1 mol/dm³, the enhancement of photolysis was distinct, shown as Fig. 4. In order to simplify the purifying of product, the concentration of phosphate was set to 0.05 mol/dm³. The photolytic solution maintained at pH 8.5.

When pure nitrogen 99.99% was bubbled for 30 minutes through the solution which contained 1.0×10⁻⁴ mol/dm³ uracil and 0.05 mol/dm³ phosphate, then the solution has been photolyzed for 15 min, the decrease of maximum absorption at 260 nm (22%) is lower than that (45%) without N₂ bubbling. From the deoxygenation experiment, we can conclude that oxygen was favourable to the "phosphate effect".

The isolation and identification of the main photoproduct of uracil

Large scale separation of photoproducts was performed using anion exchange resin. The results are presented in Fig. 5. Detailed analysis of each combined fractions showed that fraction 2 was unreacted uracil and fraction 3 and 4 were two major products.

Ultraviolet spectra of combined fraction 1 was similar to that of formic acid, which has maximum UV absorption at 240 nm. and it contained K⁺ ion, which was identified by flame reaction. Fraction 2 was unreacted uracil, which was identified by comparing the ultraviolet spectra and retention time in HPLC with authentic uracil. Fraction 5 was ortho-phosphate, which was identified by Molybdenum-blue method (Chen P.S. *et al.*, 1956). Fraction 3 was one of the photoproducts whose yield is relatively lower than that of fraction 4—product 2, and the ultraviolet absorption spectra are shown in Fig. 6. Fraction 4 was the

Fig. 1 UV spectra of photolytic nucleobases solution
A-uracil B-cytosine
1- 10^{-4} mol/dm³ nucleobases before irradiation
2-Irradiation of 10^{-4} mol/dm³ nucleobases for 15 min in the absence of phosphate
3-Irradiation of 10^{-4} mol/dm³ nucleobases for 15 min in the presence of phosphate

Fig. 2 The photolytic loss of uracil as the function of the photolysis time B: 0.05 mol/dm³ phosphate. C: no phosphate

Fig. 3 The photolytic loss of uracil as the function of the concentration of uracil in 0.05 mol/dm³ K₂HPO₄ (pH 8.5)

Fig. 4 The photolytic loss of uracil as the function of the concentration of K₂HPO₄ in 1.00×10^{-4} mol/dm³ uracil

Fig. 5 Separation of uracil and photoproducts by anion exchange resin
1-HCOOK; 2-uracil; 3-photoproduct 1; 4-photoproduct 2; 5-orthophosphate

Fig. 6 UV spectra of uracil and products
1-uracil, 2-product 1, 3-product 2

Fig. 7 Chromatogram and mass spectrum of product separated by LC/MS/MS

A- total ion chromatogram
B-mass spectrum of product

Fig. 8 UV spectra of cytosine products
1-product 1, 2-product 2

major product—6-phosphoxyuracil, which was identified by UV, $^1\text{H-NMR}$ and LC/MS/MS.

UV spectrum: From Fig. 6, the major product has λ_{max} 274 nm and 240 nm. The product having absorbing peak at 274 nm reveals that it has a conjugate system.

LC/MS/MS: Because the amount of the product was small, the purifying of it was difficult. Using LC/MS can solve this problem, which can separate the impurity before offering molecular weight. Mass fragments are obtained by tandem MS. The total ion chromatography and mass spectrum are shown in Fig. 7. The mass spectrum of product showed the molecular ion peaks at m/z 209 corresponding to $(\text{C}_4\text{H}_5\text{N}_2\text{O}_6\text{P}+\text{H})$ and exhibited a base peak at m/z 125. Other prominent peaks are observed at m/z 123, 192, 177, 163, 145, 109. The mass splitting path is explained as following:

Path A:

Path B:

$^1\text{H-NMR}$: The $^1\text{H-NMR}$ spectra of uracil and product were measured by DMX600. The sample was dissolved in D_2O . Comparing the $^1\text{H-NMR}$ of uracil (85H: 5.7 ppm, 86H: 7.4 ppm) and that of product, one hydrogen disappeared which was replaced by - OPO_3H group in product. According to LC/MS/MS, it was 6-H being replaced. 5-H was high field shifted to 8.3 ppm because of the influence of 6- OPO_3H_2 .

Lin (1996) has studied the photolysis of cytosine and isolated a main product which has been purified and identified as phosphoxycytosine ($\text{C}_4\text{H}_6\text{N}_3\text{O}_5\text{P}$) by use of ^1H -, ^{31}P -NMR spectroscopy, element analysis, ultraviolet and infrared spectroscopy and electron impact mass spectroscopy (Wang, W.Q., et al., 1999). In the present work, we found two major product of cytosine, the UV spectra are shown as Fig. 8. The second product was 6-phosphoxycytosine, which was identified by the retention time in HPLC and the UV spectra.

Mechanism of the photolysis: Since all irradiation were carried out under conditions such that 80% of the light (190-220 nm) was absorbed by the phosphate dianion, the initial process must be a transition of HPO_4^{2-} to electronically excited state $(\text{HPO}_4^{2-})^*$, then formed $\text{HPO}_4^{\cdot-}$ radical and released an electron to the solvent. This is consistent with the report (Halmann, 1966) that phosphate radical is produced by less than 220 nm UV light. The deoxygen experiment of uracil and cytosine (Lin Feng, 1996) demonstrated that O_2 is necessary for the formation of the photoproduct. According to the structures of the photoproducts and the kinetics of the photolysis reaction, a mechanism is supposed as following:

(Halmann, 1966)

Table 1. Phosphate enhancement and leucine inhibition effect of NA

NA	AMP	adeno	adeni	GMP	guano	guani	CMP	cyti	cyto	UMP	urid	ura	TMP	thyd	thymi
a1	4.32	4.88	3.15	6.12	7.35	3.70	16.2	42.9	6.25	60.0	55.8	13.6	20.0	8.25	2.74
a2	2.73	3.58	2.62	5.22	6.22	2.68	13.8	32.2	5.12	48.2	43.5	7.42	16.8	6.45	2.56
a1*	18.4	12.8	20.3	20.27	26.3	18.5	55.2	60.2	58.5	71.2	65.2	52.9	53.3	34.7	56.4
a2*	5.13	6.49	3.99	8.64	8.23	6.66	20.7	44.1	17.6	57.5	46.8	8.37	25.7	16.2	10.5

a1— photolytic loss of NA aqueous solution.

a2— photolytic loss of NA by adding 5×10^{-3} mol/dm³ of L-leucine solution.

a1*—photolytic loss of NA in 0.05 mol/dm³ K₂HPO₄ solution.

a2*—photolytic loss of NA in 0.05 mol/dm³ K₂HPO₄ by adding 5×10^{-3} mol/dm³ of L-leucine solution.

*.Abbreviations: adeno, adenosine; adeni, adenine; guano, guanosine; guani, guanine; cyti, cytidine; cyto, cytosine; urid, uridine; ura, uracil; thyd, thymidine; thymi,thymine; AMP, GMP, CMP, UMP, TMP are 5-monophosphate of adenine, guanine, cytidine, uridine and thymidine, respectively.

Phosphate enhancement and amino acids inhibition effect on photolysis of NA

Phosphate can enhance not only the photolysis of nucleobases, but also the photolysis of nucleosides and nucleotides. This phosphate effect seems to produce serious handicap. However, it can be inhibited by amino acids (Table 1). Furthermore, the inhibition effect of L-, D-leucine on the photolysis of 5'-CMP in phosphate solution shows obvious chirality difference which may be caused by the corresponding stereochemical affinity between L-leucine and D-sugar constituted in 5'-CMP (Pan, *et al.*, 1995).

Conclusion

1. Phosphate can enhance the far UV photolysis of nucleobases, nucleosides and nucleotides.
2. The photoproduct 6-phosphoxyuracil is a novel compound which has been identified by UV, ¹H-NMR, LC/MS/MS.
3. A reasonable photolysis mechanism of uracil in phosphate solution has been suggested, which seems to have shown promise for phosphorylating nucleic acid monomers.

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