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The comparative investigation of the dehydration methods of uracil hydrate derivatives

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Abstract

By following UV absorbance, UV absorption spectra and changes in high performance liquid chromatography hydrate peak elution areas during the reversion of uracil, uridine and 5′UMP hydrates induced by heating hydrated samples in boiling water for 10 min or by incubating acidified samples (pH 1) at room temperature for 24 h, it is shown that these methods do not lead to quantitative dehydration. The disappearance of hydrate peaks and the higher recovery of both the UV absorbance and UV absorption spectra obtained after heating acidified samples (pH 1) indicate that this method is best to achieve total dehydration. Catalysis of dehydration in phosphate buffer solution is also discussed.

Keywords: Uracil; Uridine; 5′UMP; Hydrates; Dehydration

1. Introduction

The formation and the properties of the photohydrates have been extensively reviewed [1–3]. A means of quantifying the photohydrates' formation is that the photolysis of some pyrimidines, such as uracil, uridine and UMP, can be reversed by subsequent acidification or heating [4,5]. These methods have generally been accepted as being equally effective for dehydration. However, an obvious difference in the extent of dehydration between heating irradiated uridine and 5′UMP aqueous solutions and heating those containing 0.1 M phosphate buffer solution (PBS) was noted during investigation of the enhancement of the photolysis of nucleic acid monomers by phosphates [6,7].

2. Experimental

In our experiments we have employed a 9 W low pressure mercury lamp as a radiation source. A 20% acetic acid solution of 1 cm pathlength was employed to filter off the photochemically significant quantities of 184.9 nm, 194.2 nm and 222.4 nm UV light. For actinometry we used the chromophore loss of uridine in aerated aqueous solution at pH 6 ($\phi_{cl}=0.018$) [8,9]. Light intensity at irradiated site is 0.62 Einstein min^{-1}. Dehydration methods used were those introduced by Sinsheimer and Hasting [4,5] and by Moore and Thomson [10,11]. Uracil, uridine and 5′UMP (Sigma Co.) solutions were irradiated directly in quartz cells (Beckman). Substrate concentration was of the order of 1×10^{-4} M, and chromophore changes during irradiation were followed using a Beckman DU 640 spectrophotometer. For comparison of the dehydration efficiencies of different dehydration methods, irradiated samples were mixed and then diluted to half of their initial concentrations so as to facilitate the addition of PBS (Na_2HPO_4, KH_2PO_4, pH 6.98) and hydrochloric acid. The reversal percentage is defined as the percentage of UV absorbance at λ_{max} of uracil, uridine and 5′UMP after dehydration compared with the decrease in absorbance after irradiation. In the previous investigations, quantification of hydrate formation was obtained by following absorbance changes and no attempts were made to determine whether total dehydration had been achieved in the solutions studied. Wang noted [12] that reactions of the hydrates with only acid, alkaline or heat did not bring about a total reconstitution, but his attempt to isolate the product from the reaction mixture was not successful. In this experiment uracil and uridine were distinguished from their hydrates based on the modified methods introduced by Gurzadyan and Gorner [8,9] (ODS, Hypersil 200×4.6 mm, 2 ml min^{-1} for uridine and 1 ml min^{-1} for uracil, detected at 200 nm and 262 nm, Hewlett Packard 1050 series). The assignment of high performance liquid chromatography (HPLC) elution peaks corresponding to starting material and

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photohydrates was supported by results that after dehydration of irradiated samples the hydrate peaks disappeared and the initial substrate was recovered. Hydrate peaks of 5'UMP can not be separated from their parent under the present conditions. The eluent was prepared from a Millipore (Milli Q) system. No further additives were used in elution process because of their absorbance at 200 nm.

3. Results and discussion

Dehydrate results of the photohydrates of uracil, uridine and 5'UMP are summarized in Tables 1 and 2. Although it has been claimed that heating irradiated samples in a boiling water bath for 15 min can lead to an increase in absorption comparable with that produced by addition of hydrochloric acid [5], we have shown that total dehydration can not be achieved either by heating samples in boiling water for 10 min or by incubating acidified samples at room temperature for 24 h. The reversal percentage obtained by heating samples in a boiling water bath for 10 min is the lowest among the results obtained. The typical absorption spectra of the hydrates (below 220 nm) after heating samples for 10 min can be clearly observed (Fig. 1). HPLC elution of the heated samples also clearly demonstrates the residual peaks of uridine hydrate isomers (Table 3). In the recent works by Gurzadyan and Gorner [8,9], the identification of the hydrates was based on the disappearance of the hydrate peak after heating samples from 5 min to 30 min and the recovery of the initial substrate. From Table 4 it is clear that quantitative dehydration can not be achieved by heating the samples even for 30 min.

The reversal percentage obtained by incubating acidified samples (pH 1) for 24 h in the dark at room temperature [4,5] is higher than that of the above method from Tables 1–3. Elution peaks corresponding to hydrates of uracil and uri-

Table 1
Comparison of reversal percentage using different dehydration methods

NA	Time (min)	A_0	A_t	Additive	R_1	R_2	R_3
Urd	10	1.087	0.264	–	31.8	8.0	54.7
				PBS	86.5	20.4	93.1
				pH 1	96.5	70.5	98.4
	60	1.020	0.008	–	77.3	5.7	63.0
				PBS	97.2	17.8	97.6
				pH 1	98.8	69.0	101
	180	1.040	0.021	–	21.7	18.0	88.8
				PBS	91.8	26.6	93.1
				pH1	95.7	69.2	98.0
	720	1.032	0.027	–	14.0	12.0	40.9
				PBS	62.0	27.2	62.6
				pH 1	64.4	44.1	63.4
UMP	20	1.025	0.064	–	40.4	6.5	61.4
				PBS	74.9	13.1	84.1
				pH 1	100	95.7	103
	60	1.000	0.001	–	68.8	8.3	71.4
				PBS	79.0	11.9	89.7
				pH 1	99.8	93.0	103
	180	1.030	0.033	–	53.9	11.7	69.9
				PBS	70.9	17.0	83.8
				pH 1	93.2	85.4	92.6
	720	1.029	0.020	–	19.6	17.0	58.9
				PBS	62.6	25.2	62.6
				pH 1	66.4	60.1	65.4
Ura	80	0.869	0.452	–	20.6	11.0	12.9
				PBS	64.3	15.8	50.4
				pH 1	48.9	36.9	52.3
	180	0.849	0.081	–	17.1	13.7	54.6
				PBS	50.9		50.4
				pH 1	53.3		47.0
	720	0.857	0.006	–	1.9	3.5	5.9
				PBS	33.8	14.8	39.7
				pH 1	35.7	33.8	34.5

Reversal percentage is calculated on the basis of the equation $(A_H - A_t/A_0 - A_t) \times 100\%$, where A_0 is the initial absorbance at λ_{max} of uracil, uridine and 5'UMP, A_t is the absorbance after irradiation; A_H is the absorbance after dehydration. R_1 is the result obtained by heating samples in a boiling water bath for 10 min directly after irradiation, R_2 is the result for samples after 24 h of incubation in the dark at the room temperature and R_3 is the result by heating of samples in boiling water bath for 10 min after 24 h of incubation in the dark at room temperature.

Table 2
Reversal percentage by different methods versus irradiation time

Irradiation time (min)	5	10	15	20	30	40	50	60
A_0 (Urd)	1.074	1.074	1.075	1.074	1.075	1.078	1.078	1.078
A_t (Urd)	0.644	0.371	0.101	0.066	0.021	0.018	0.018	0.016
R_1 (Urd)	42.3	40.5	50.2	25.4	43.7	30.8	33.0	48.2
R_2 (Urd)	65.6	70.1	72.2	70.8	70.7	69.8	71.9	71.8
R_3 (Urd)	96.7	100	102	101	101	98.2	101	101
A_0 (UMP)	1.051	1.050	1.047	1.049	1.048	1.046	1.036	1.035
A_t (UMP)	0.655	0.350	0.189	0.059	0.020	0.013	0.014	0.017
R_1 (UMP)	54.8	60.9	65.9	66.2	68.5	69.0	69.7	73.4
R_2 (UMP)	92.2	91.1	92.9	91.4	91.2	91.3	89.8	89.5
R_3 (UMP)	102	101	103	101	101	100	99.4	99.1

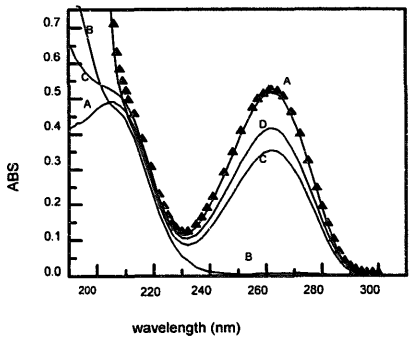

Fig. 1. UV spectra of uridine (A) before and (B) after 60 min of irradiation. Curves (C) and (D) are absorption spectra of the dehydrated samples obtained by heating the aqueous solution at 100 °C for 10 min and by incubating at pH 1 for 24 h. Triangles on curve (A) are absorption spectra of the dehydrated samples obtained by heating the acidified solution (pH 1) in a boiling water bath for 10 min.

dine isomers were clearly observed during HPLC elution of acidified samples (Table 3). Acid-catalyzed dehydration was found to follow first-order kinetics with respect to the hydrates of uridine, 1,3-dimethyluracil and 5′UMP. During acid reversion the rate constant at pH 1 is 55.5×10^{-3} min^{-1} for the hydrates of uridine within about 60 min, after which the rate constant decreases markedly [13]. Similar results was also achieved during acid reconstitution of 1,3-dimethyluracil hydrates [13]. Therefore it is theoretically insufficient to expect total dehydration of uridine hydrates in 24 h. It may be concluded that this is not an appropriate approach to total dehydration in spite of the fact that it has been cited as a standard method for dehydration by some textbooks e.g. Ref. [14].

Catalysis of dehydration by the phosphate group of 5′CMP has been suggested to account for the sixfold increase in reversal rate compared with that of 3′CMP [3]. From Table 1 we can see that catalysis in phosphate buffer solution is significant for uracil and its derivatives, but the rate increase is extremely slow at room temperature. The reversal percentage may be accelerated by raising the temperature (Tables 1 and 2). Addition of PBS before or after irradiation does not affect the reaction process. Also, the dehydration efficiency does not appear to be dependent on the PBS concentration in the range from 1×10^{-3} M to 0.5 M. From Tables 1 and 2 it is evident that the efficiency of dehydration by heating samples containing PBS is still lower than that of heating acidified samples. Combined with the observations of residual elution area of uracil and uridine hydrate peaks in HPLC chromatograms (Table 3), this indicates that heating hydrate solutions containing PBS is not the best way to achieve total dehydration.

Table 3
Elution peak areas of uridine, uracil and their hydrates using different dehydration methods

	RT	A_0	$A_{t(60\,min)}$	H_1	H_2	H_3	S_1	S_2	S_3
$Abs_{(262\,nm)}$	–	1.080	0.049	0.414	1.088	1.102	0.086	0.234	0.862
Urd	4.20	518	0	196	516	530	16	94	402
Hydrate 1	1.59	0	255	181	14	0	252	236	61
Hydrate 2	1.96	0	284	206	10	0	288	254	67
$Abs_{(259.5\,nm)}$	–	0.899	0.472	0.648	0.666	0.652	0.516	0.534	0.636
Ura	3.18	1121	659	771	742	998	692	713	961
Hydrate	2.65	0	197	41	0	0	132	97	21

RT: retention time (min); This table shows the elution area of samples after heating in boiling water for 10 min (H_1) or heating those containing 0.1 M PBS (H_2) or 0.1 M HCl (H_3). S_1 indicates the elution area of sample after incubating for 24 h, S_2 and S_3 are the elution areas of samples containing 0.1 M PBS and 0.1 M HCl respectively after incubating for 24 h.

Table 4
Reversal percentage as a function of heating time. Reversal percentage is obtained by prolonged heating of irradiated uridine and 5'UMP samples

NA	A_0	Time (min)	A_t	5 min	10 min	15 min	20 min	25 min	30 min	$H^*_{10\,min}$
Urd	1.056	20	0.046	13.3	24.4	36.8	47.7	57.1	65.9	102
UMP	1.061	20	0.084	37.1	63.6	75.6	83.5	89.3	92.5	100

$H^*_{10\,min}$: reversal percentage obtained by firstly acidifing the heated (30 min) samples to pH 1 then heating acidified samples for 10 min in a boiling water bath.

From Tables 1–4 it is clear that the efficiency of dehydration by heating acidified samples immediately or after incubating for 24 h is always higher than that of other methods employed. Total reversion of the initial UV spectra, UV absorbance and the disappearance of hydrate peaks after heating acidified samples suggest that this is the best approach to total dehydration. The relatively low reversal percentage of uracil, uridine and 5'UMP samples after prolonged irradiation is due, of course, to eventual destruction of initial photoproducts.

We can see from Tables 1–3 that hydrates are the dominant products of uridine within 3 h of irradiation. From changes of UV absorbance, UV absorption spectra and HPLC elution areas after heating acidified samples we can estimate that the reversal percentage is close to 100% if the irradiation time is less than 3 h. The similar recovery in both UV absorbance and UV spectra seems to indicate that hydration is also the dominant process during irradiation of 5'UMP. For uracil, the elution area of its hydrate accounts for only about half of its decreased substrate elution area.

As to the quantification of hydrate formation, heating acidified samples may be taken as a method of quantifying the hydrates of uridine, and possibly for 5'UMP and uracil only under the condition of using 0.1 mM or lower concentration solutions and conducting irradiation under air because of the reported limiting concentrations for uracil dimerization (>0.1 mM) and oxygen quenching the formation of pyrimidine dimers [1–3,15]. In view of the instability of anti cyclobutane dimers of uracil and uridine with respect to reversion to parent compound upon heating in acid [2,3,16] and the possibly of other photoproducts during acid reversion, it should be emphasized that there are possibly problems with using reversal achieved by heating in acid as the sole quantitative measurement of hydrate formation without accompanying experiments being undertaken. The preferred supplementary method for quantifying hydrate formation would be chromatography.

Acknowledgements

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