JOURNAL OF PHYSICAL ORGANIC CHEMISTRY
J. Phys. Org. Chem. 2002; **15:** 293–305
Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/poc.478

Kinetic and spectral investigation of the electron and hydrogen adducts of dihydroxy- and dimethyl-substituted pyrimidines: a pulse radiolysis and product analysis study

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Received 23 July 2001; revised 29 November 2001; accepted 4 January 2002

epoc ABSTRACT: The reactions of hydrated electrons (e_{aq}^-) and hydrogen atoms ($H^\cdot$) with 4,6-dihydroxy-2-methylpyrimidine (DHMP), 2,4-dimethyl-6-hydroxypyrimidine (DMHP), 5,6-dimethyluracil (DMU) and 6-methyluracil (MU) were studied at different pH values using pulse radiolysis. The second-order rate constants obtained for the reaction of e_{aq}^- with these systems are in the range (5–10) $\times 10^9$ dm^3 mol^{-1} s^{-1} at near, neutral pH. At basic pH, the rate constant values were considerably reduced owing to the electrostatic effect between e_{aq}^- and pyrimidine anion. The transient absorption spectra of the electron adducts have distinct absorption maxima at around 300–320 nm. The initial spectrum in the case of DHMP at pH 4.5 was found to undergo a first-order transformation. Based on the spectral characteristics and the yields of methylviologen radical cation ($MV^{\cdot+}$) resulted from the electron transfer reaction between the electron adducts and MV^{2+}, it is proposed that a protonated (at oxygen) electron adduct of DHMP is initially formed which undergoes a proton- and phosphate-catalysed transformation to form a reducing C(5) protonated C(6)-yl radical. Such preferential protonation at C(5) is predominant only with dihydroxypyrimidine systems. At pH 9 and 13, formation of a radical monoanion of DHMP ($pK_a \geq 13$) is proposed. The possible attack of e_{aq}^- is proposed to be at N(1) or N(3) of DMHP. The resulting electron adduct has a pK_a value around 6.0. Similar properties for the electron adducts of DMU and MU [electron attack at O(4)] are proposed. The second-order rate constants for $H^\cdot$ with DHMP, DMHP, DMU and MU were in the range (1.7–28) $\times 10^8$ dm^3 mol^{-1} s^{-1}. The hydrogen adduct spectra were generally identified as their absorption maxima at 310–380 and 460–510 nm. Formation of C(5)-protonated C(6)-yl radical, the same radical that formed after the H^+-and phosphate-catalysed transformation of the electron adduct, is proposed for DHMP. The possibility of the formation of C(5)-yl and C(6)-yl H adducts of DMHP, DMU and MU is discussed. High-performance liquid chromatography coupled with electrospray mass spectrometry (HPLC-ES-MS) has been used to qualitatively analyse the products obtained from the reaction of e_{aq}^- with DHMP and DMHP and the results revealed that the products are mainly derived from the C(5) protonated C(6)-yl radicals via its disproportionation and dimerization reaction. A possible reaction mechanism is proposed for the product formation. Copyright © 2002 John Wiley & Sons, Ltd.
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KEYWORDS: pulse radiolysis; pyrimidines; electron adducts; hydrogen adducts; electrospray mass spectrometry

INTRODUCTION

Radiation chemical studies of DNA model systems such as nucleobases and nucleosides are important in understanding the chemical basis of radiation-induced lesions in DNA. Several reports[1–7] on radiation chemical studies of purines and pyrimidines are available and most of these studies are directed to the understanding of the reactions of water-derived free radicals with DNA model systems. Such studies are carried out in dilute aqueous solutions where the radiolysis of water provides the primary free radicals:

$$H_2O \rightsquigarrow {}^\cdot OH, e_{aq}^-, {}^\cdot H, H_3O^+, H_2O_2, H_2 \qquad (1)$$

The reactions of hydroxyl radical ($^\cdot$OH) and hydrated electron (e_{aq}^-) with purines, pyrimidines and their nucleosides are often very fast and are diffusion controlled.[8] The formation of electron adducts and their

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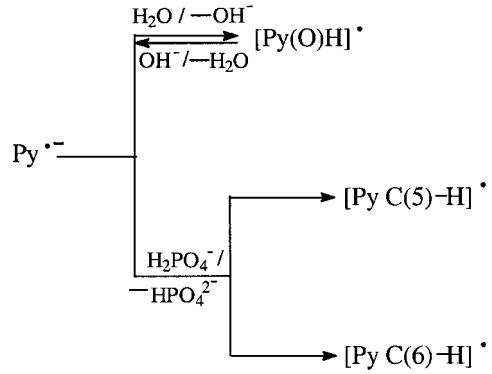
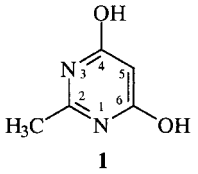
Contract/grant sponsor: Board of Research in Nuclear Sciences (BRNS), Government of India.

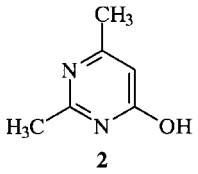


Scheme 1



1

4,6- dihydroxy 2-methyl pyrimidine (DHMP)



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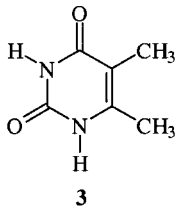
2, 4-dimethyl 6-hydroxy pyrimidine (DMHP)



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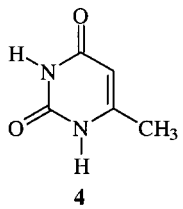
5,6- dimethyl uracil (DMU)



4

6- methyl uracil (MU)

fast protonation ($k \geq 10^7$ s^{-1}) at heteroatoms in the solution state have been reported with purine bases (e.g. adenine, guanine and hypoxanthine).[9–13] The conversion of the initially formed heteroatom-protonated electron adducts into carbon-protonated electron adducts is predominant (at least on the pulse radiolysis scale) only in purine nucleosides.[10–14] These transformation reactions are catalysed by both OH^- and phosphate buffer. However, the rates of protonation at the carbon site of various nucleosides were different, owing to the substituent effect. Both kinetic and thermodynamic factors are known to control this transformation phenomena, as can be seen in several recent reports.[10–14]

On the other hand, the protonation of the initially formed electron adducts of pyrimidines occurs at different rates in the solution state. For example, protonation of the electron adduct of cytosine by water is reported to be considerably fast ($k = 3.5 \times 10^6$ s^{-1})[14] but that of uracil derivatives is relatively slow ($k < 5 \times 10^5$ s^{-1}).[15–17] In the case of uracil and its methyl-substituted derivatives, the conversion of the oxygen-protonated electron adduct to carbon-protonated electron adduct [C(6) protonation] can be catalysed by phosphate buffer (Scheme 1). However, the protonation at C(6) was observed to depend on the site of methyl substitution in the pyrimidine ring and the rate constants varied from 1.6×10^7 dm^3 mol^{-1} s^{-1} for 1,3-dimethylthymine to $<10^5$ dm^3 mol^{-1} s^{-1} for 6-methyluracil.[16,17] The oxygen-protonated and the carbon-protonated radicals can be differentiated from their redox behaviour using the oxidants methylviologen (MV^{2+}), *p*-nitroacetophenone (pNAP) or tetranitromethane (TNM).[17]

Although both C(5) and C(6) are potential sites of protonation, it is generally observed that C(6) protonation is more favoured in the case of thymine, uracil and cytosine.[17] On the other hand, C(5) protonation catalysed by H^+, phosphate and OH^- was reported to be more favoured in the case of 4,6-dihydroxypyrimidine, based on an ESR study in the liquid state.[18] It is important to note that the preferential protonation at C(5) catalysed by H^+, phosphate or OH^- has not yet been clearly established by pulse radiolysis studies of any pyrimidine derivatives. Therefore, we selected methyl-substituted dihydroxypyrimidine, considered as an isomer of cytosine, along with dimethylhydroxypyrimidine, dimethyluracil and methyluracil, and studied the possibility of transformation reactions at different concentrations of H^+, OH^- and phosphate. The effect of substituents and their position in the pyrimidine ring on the transformation reaction was investigated. Furthermore, it will be shown that 4,6-dihydroxy-2-methylpyrimidine is an ideal system to demonstrate the preferential protonation at the C(5) position using the oxidant, methylviologen (MV^{2+}), which is not a major process with the electron adducts of other pyrimidine derivatives reported so far. The selected systems were 2,4-dimethyl-6-hydroxypyrimidine (DMHP), 4,6-dihydroxy-2-methylpyrimidine (DHMP), 6-methyluracil (MU) and 5,6-dimethyl uracil (DMU), and the structures **1–4** represent the neutral forms of these pyrimidines. The kinetic and spectral parameters at different pH values are also determined. The oxidant MV^{2+} was used to investigate the redox nature of the electron adducts.

The end product analysis after the reaction of water-derived free radicals with biomolecules is often a difficult task owing to the low concentrations of the products [$(1–10) \times 10^{-6}$ mol dm^{-3} in low-dose experiments]. However, considerable progress was made in this field with the help of gas chromatography–mass spectrometry in the case of OH radical reactions with purines and pyrimidines.[19] On the other hand, very little information is available about the end products of e_{aq}^- reaction with purines and pyrimidines. Therefore, an attempt was made to analyse qualitatively the end products of the reaction of e_{aq}^- with DHMP and DMHP using high-performance liquid chromatography coupled with electrospray mass spectrometry (HPLC–ES-MS).

Table 1. Second-order rate constants (k) for the reaction of e_{aq}^- with DHMP, DMHP, DMU and MU, the yields $G(MV^{\bullet+})$ from the reaction of their protonated electron adducts with MV^{2+} and the second-order rate constants (k') obtained at different pH values[a]

Compound	pH	k ($dm^3\ mol^{-1}\ s^{-1}$)	$G(MV^{\bullet+})$[b]	k' ($dm^3\ mol^{-1}\ s^{-1}$)
DHMP	4.5	5.4×10^9	2.7	1.4×10^{10}
	9.0	3.1×10^9	2.7	1.0×10^{10}
	13.0	2.0×10^9	3.1	1.0×10^{10}
DMHP	6.0	1.3×10^{10}	2.5	1.1×10^{10}
	12.5	1.0×10^9	2.6	1.4×10^{10}
DMU	6.0	1.1×10^{10}	2.7	1.0×10^{10}
	12.0	1.7×10^9	2.8	1.1×10^{10}
MU	6.0	1.3×10^{10}	2.6	8.0×10^9
		(1.0×10^{10})[c]	3.3[d]	6.0×10^9

[a] $k' = k$ (electron adduct + MV^{2+}) (these were determined from the pseudo-first-order buildup of $MV^{\bullet+}$ from a single concentration of MV^{2+} and hence are approximate).
[b] These are averages of at least three values.
[c] From Ref. 8.
[d] At pH 12.

EXPERIMENTAL

Commercially available DHMP, DMHP, MU and DMU, obtained from Aldrich, were used without further purification. Solutions were prepared in water obtained from a Millipore Milli-Q purification system. The concentrations of the substrate were usually maintained at 1×10^{-3} mol dm^{-3}. Pulse radiolysis experiments were carried out using a linear accelerator delivering electron pulses of 7 MeV energy of 50 ns duration. An aerated aqueous solution of KSCN (1×10^{-2} mol dm^{-3}) was used to monitor the dose per pulse with $G \times \epsilon_{500} = 21\,520$ $dm^3\ mol^{-1}\ cm^{-1}$ and was normally kept at 14–15 Gy. A low dose per pulse of 5.5 Gy was used for the investigation of the electron transfer reaction between the electron adducts and MV^{2+}. The transient species formed on pulse radiolysis were monitored by using a 450 W pulsed xenon arc lamp, a monochromator (Kratos GM-252) and a Hamamatsu R-955 photomultiplier as the detector. The photomultiplier output was digitized with a 100 MHz storage oscilloscope interfaced to a computer for kinetic analysis. The rate values are the averages of at least three experiments and the variation was within 15%. The rate constant values were taken from those kinetic analyses for which a very good correlation was obtained between the experimental and calculated results.[20] The details of the pulse radiolysis set-up have been described elsewhere.[20–22]

The radiolysis of N_2-saturated neutral water produces three highly reactive species (e_{aq}^-, $^{\bullet}OH$ and $H^{\bullet}$) in addition to the formation of inert or less reactive molecular products (H_2 and H_2O_2) [reaction (1)]. The reactions of e_{aq}^- were investigated in N_2-saturated aqueous solutions containing either 2-methylpropan-2-ol or propan-2-ol for scavenging $^{\bullet}OH$ and $G(e_{aq}^-) = 2.7$ is expected at neutral pH.

The reaction of H atoms was investigated at neutral pH with $G(H^{\bullet}) = 0.6$ in N_2O-saturated solutions in presence of 2-methylpropan-2-ol as an $^{\bullet}OH$ scavenger:

$$(CH_3)_3COH + {}^{\bullet}OH \longrightarrow {}^{\bullet}CH_2(CH_3)_2COH \qquad (2)$$

At pH 1, $G(H^{\bullet}) = 3.3$ as e_{aq}^- is converted to $H^{\bullet}$:

$$e_{aq}^- + H^+ \longrightarrow H^{\bullet} + H_2O \qquad (3)$$

Qualitative product analysis was carried out using HPLC–ES-MS after γ-radiolysis of an Ar-saturated solution containing either DHMP and DMHP (1×10^{-3} mol dm^{-3}) in presence of 2-methylpropan-2-ol (0.3 mol dm^{-3}) with an absorbed dose of 2000 Gy. The mass spectra were recorded for both positive and negative ions in the range 50–1000 *m/z* units at 3 s per scan and a skimmer voltage of 3.5 kV. A 3 m, 100×4 mm i.d. Hypersil BDS C_{18} column (Hewlett-Packard) and Millipore Milli-Q water as eluent (flow-rate = 0.5 $dm^3\ min^{-1}$) were used.

RESULTS AND DISCUSSION

Acid–base properties

DHMP is reported to have three different pK_a values (0.21, 6.35 and 12.9) and exists predominantly in the monoketo–monoenol form.[23] The pK_a values of DHMP were determined experimentally, using UV–VIS spectrophotometry, as 3.15 and 9.9. Two pK_a values (2.7 and 9.8) were determined in the case of DMU. The reported pK_a value of MU is 9.52[24] and the experimentally determined value was 9.5.

Reactions of hydrated electron

Kinetic parameters. The second-order rate constants of

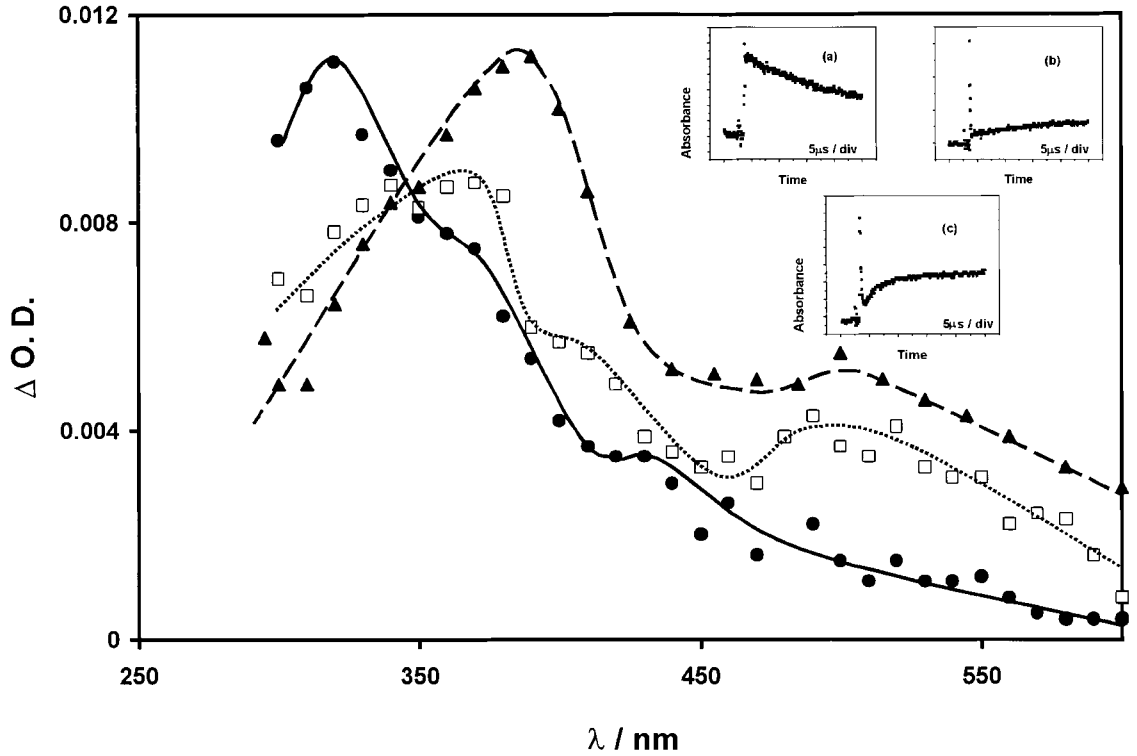


Figure 1. Transient absorption spectra recorded in N_2-saturated aqueous solutions of DHMP (1×10^{-3} mol dm^{-3}) containing propan-2-ol (0.2 mol dm^{-3}) at 3 μs (●) and 40 μs (□) after the pulse at pH 4.5 and the spectrum recorded in the presence of phosphate buffer (0.3 mol dm^{-3}) at 15 μs (▲) after the pulse at pH 7.5; dose per pulse ≈ 16.5 Gy. Inset: traces of the intermediates obtained at 320 nm (a) and 490 nm (b) in the absence of phosphate and at 485 nm (c) in the presence of phosphate

the reaction of hydrated electrons with DHMP, DMHP, DMU and MU in N_2-saturated aqueous solutions containing either 0.2 mol dm^{-3} 2-methylpropan-2-ol or propan-2-ol to scavenge OH radicals were determined by following the decay of the hydrated electron at 720 nm as a function of substrate concentration. The pseudo-first-order decay constant (k_{obs}) versus concentration plot was a straight line with very good correlation coefficients ($\geq$0.99). The rate constants of the neutral forms are diffusion controlled (Table 1) and these values are in agreement with the rate constants reported for other pyrimidines.[8] However, at higher pH values where the pyrimidines are in their monoanionic form, the rate constant values were significantly reduced except in the case of DHMP, where the difference was only marginal. The lowering of rate constants at higher pH values, as shown in Table 1, represents the electrostatic effects resulting from the pyrimidine anion and hydrated electron. Similar trends in the rate constant values were reported with mono- and dianionic forms of both purines and pyrimidines.[6]

Spectra. *4,6-Dihydroxy-2-methylpyrimidine (DHMP).* The time-resolved absorption spectra obtained at pH 4.5 from the reaction of e_{aq}^- with DHMP are shown in Fig. 1. The spectrum recorded at 3 μs after the pulse has an absorption maximum at 320 nm with a shoulder at around 380 nm. While this spectrum showed a decay at 320 nm ($k = 5.5 \times 10^4$ s^{-1}), a further increase in absorbance was observed above 350 nm ($k_{490\ nm} = 4 \times 10^4$ s^{-1}) indicative of a transformation of the initial species. As can be seen from Fig. 1, the transformed spectrum has λ_{max} around 380 nm and a broad absorption between 460 and 550 nm. A typical trace obtained at 490 nm is shown in the inset of Fig. 1. The time-resolved spectra were also recorded in the presence of phosphate buffer (0.3 mol dm^{-3}) at pH 7.5. It was observed that the decay at 320 nm and the build-up above 350 nm are greatly enhanced ($k_{380\ nm} = 3.6 \times 10^5$ s^{-1}, $k_{490\ nm} = 2.0 \times 10^5$ s^{-1}). The transformed spectrum measured at 15 μs after the pulse and typical traces at 380 and 490 nm are shown in Fig. 1. The spectral characteristics at pH 9 were different from those at pH 4.5. The observed first-order decay at 320 nm and the slow build-up of absorbance above 350 nm at pH 4.5 were not visible at pH 9, but the spectrum showed two distinct maxima at 310 and 360 nm. Similarly, the spectrum recorded at 3 μs after the pulse at pH 13 (Fig. 2) was found to decay without showing any delayed build-up, unlike at lower pH.

In order to understand the nature of the electron adducts, we investigated the electron transfer reaction of

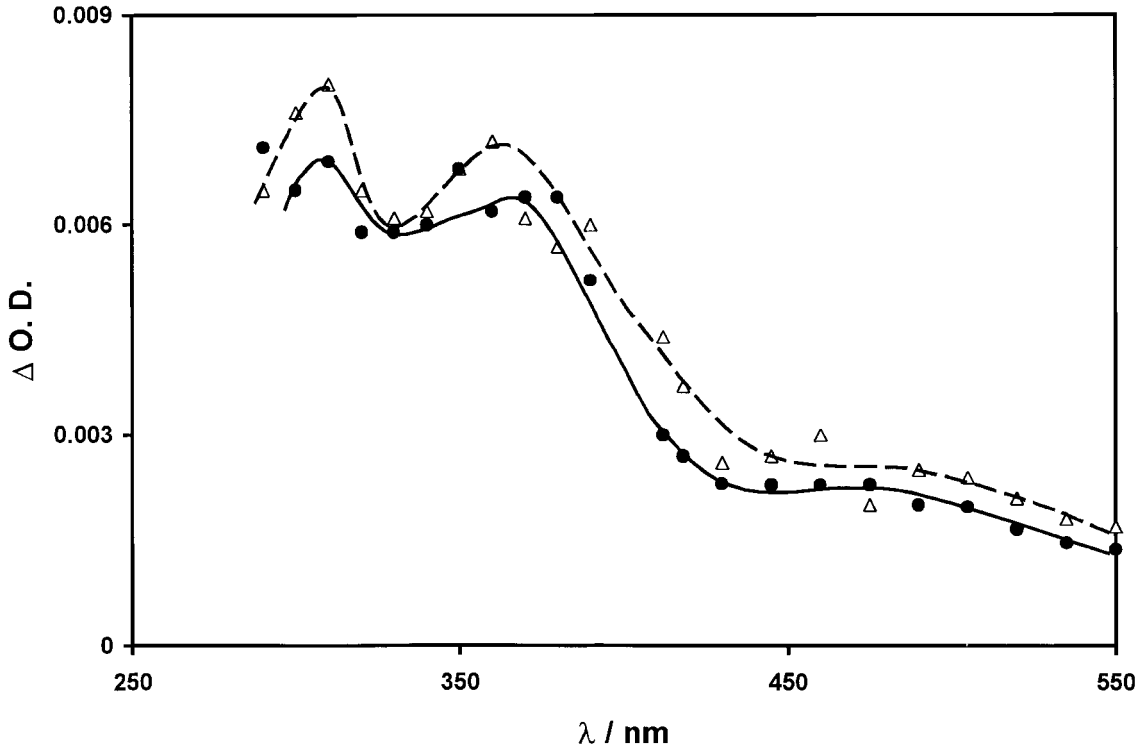
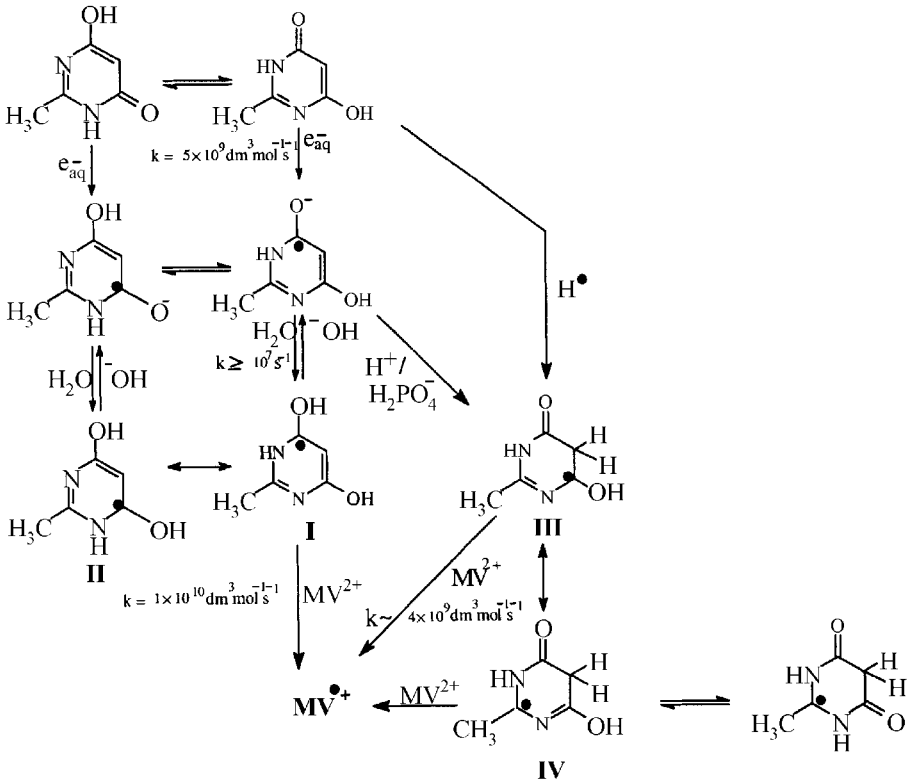


Figure 2. Transient absorption spectra recorded in N_2-saturated aqueous solutions of DHMP (1×10^{-3} mol dm^{-3}) containing 2-methylpropan 2-ol (0.2 mol dm^{-3}) at 3 μs after the pulse at pH 9 (△) and pH 13 (●); dose per pulse ≈ 16 Gy

the electron adducts with MV^{2+} at different pH values. The formation of methylviologen radical cation ($MV^{\cdot+}$) was monitored at 605 nm. 2-Methylpropan-2-ol was used as the OH radical scavenger in all these experiments to avoid the reaction of 2-hydroxy-2-propyl radical, formed from the reaction of the OH radical with propan-2-ol, with MV^{2+}. The yields, $G(MV^{\cdot+})$, and the second-order rate constants for the electron transfer reaction are compiled in Table 2. The G values were calculated by taking a typical ε value of $MV^{\cdot+}$ as 12 800 dm^3 mol^{-1} cm^{-1} at 605 nm.[25] The G value of $MV^{\cdot+}$ obtained at pH 4.5 was 2.7. Expecting about 20% of the direct reaction of e_{aq}^- with MV^{2+} under these experimental conditions {$[MV^{2+}] = 5 \times 10^{-5}$ mol dm^{-3}, $[DHMP] = 2 \times 10^{-3}$ mol dm^{-3}, $k(e_{aq}^- + MV^{2+}) = 5.4 \times 10^{10}$ dm^3 mol^{-1} s^{-1}}, the observed $G(MV^{\cdot+})$ corresponds to the total yield of the electron adduct. Similarly at pH 9, the observed G value of 2.7 indicates the quantitative reaction of the electron adduct with MV^{2+}. At pH ≈ 13, a $G(MV^{\cdot+})$ value of 3.1 is obtained. At this pH, a considerable part of $H^{\cdot}$ will be converting into e_{aq}^- [$k(H^{\cdot} + OH^-) = 2.3 \times 10^7$ dm^3 mol^{-1} s^{-1}].[4] Calculating the yield of e_{aq}^- at this pH as 3.1, the observed $G(MV^{\cdot+})$ indicates a quantitative reaction of the electron adduct.

The attack of e_{aq}^- is likely to occur at the electron affinic oxygen, O(4) or O(6) of the keto form. The resulting radical anion (ketyl radical) can be protonated by water. The nature of the initial spectrum recorded 3 μs after the pulse at pH 4.5 with a λ_{max} at 320 nm is in agreement with the electron adduct spectra of other pyrimidines reported earlier.[16,17,25] We propose that the spectrum absorbing at 320 nm corresponds to the protonated electron adduct of DHMP as the protonation of the electron adducts of pyrimidines at oxygen is normally very fast with pK_a values around 7.2.[25] Moreover, the feasibility of protonation at two identical C(4) and C(6) oxygen (as shown in Scheme 2) makes the protonation more efficient compared with the uracil electron adduct. Therefore, in the present case it is likely that the radical anion must become protonated with a half-life <1 μs (this is the minimum time required to measure the initial spectrum using our experimental set-up) and the proposed structure is **I** (Scheme 2) which is in resonance with **II**. Such fast protonation is justified based on earlier reports on the protonation at oxygen or nitrogen in the case of uracil derivatives[5–17] ($k \approx 5 \times 10^5$ s^{-1}) and cytosine[14] ($k \approx 3.5 \times 10^6$ s^{-1}). The first-order nature of the decay of the initial spectrum at its λ_{max} and the corresponding delayed build-up at $\lambda \geq 350$ nm indicate a clear first-order transformation of the initially formed protonated electron adduct to a different species with a λ_{max} around 380 nm and a broad absorption band between 490 and 550 nm. It is interesting that the nature of this transformed spectrum and the spectrum recorded 15 μs after the pulse in the presence of phosphate buffer (0.3 mol dm^{-3}) are very similar (Fig. 1), which implies that the transformation can be catalysed by both H^+ and



Scheme 2

phosphate. However, the rate of transformation was almost 10 times higher in the presence of phosphate buffer. A phosphate-catalysed conversion of oxygen-protonated electron adduct to carbon-protonated [C(5) and C(6)] electron adduct is a well established reaction in the case of uracil, thymine and some of their substituted derivatives.[16,17] On the other hand, it appears that such transformations catalysed by H^+ are not very common, but restricted to only dihydroxypyrimidines as reported in an ESR study by Novais and Steenken.[18] Therefore, we can clearly understand that the transformed species is a carbon-protonated electron adduct of DHMP. Since such carbon protonation can be at C(5) and/or C(6), as observed with uracil and thymine,[16] one has to estimate clearly the percentage distribution of these protonated radicals. The results obtained with MV^{2+} gave a detailed account of the contribution of reducing radicals. The observed G value of $MV^{\cdot+}$ (i.e. 2.7) at pH 4.5 clearly indicates that the protonated electron adduct is reducing in nature. This is understandable from the proposed structure of the protonated electron adduct (**I**) in which the unpaired spin density is at carbon. This is in agreement with the earlier reports with uracil and its derivatives.[16,17] The G of $MV^{\cdot+}$ further leads to the conclusion that even the transformed species at pH 4.5 is reducing in nature. If we assume that the transformed protonated electron adduct ($k_t = 4 \times 10^4\ s^{-1}$) is a non-reducing radical (as reported with uracil and thymine[16]), one can calculate the G value of $MV^{\cdot+}$ [Eqn. (1)] from the competition between the reaction of electron adduct (EA) with MV^{2+} [reaction (4)] and the transformation of the electron adduct [reaction (5)].

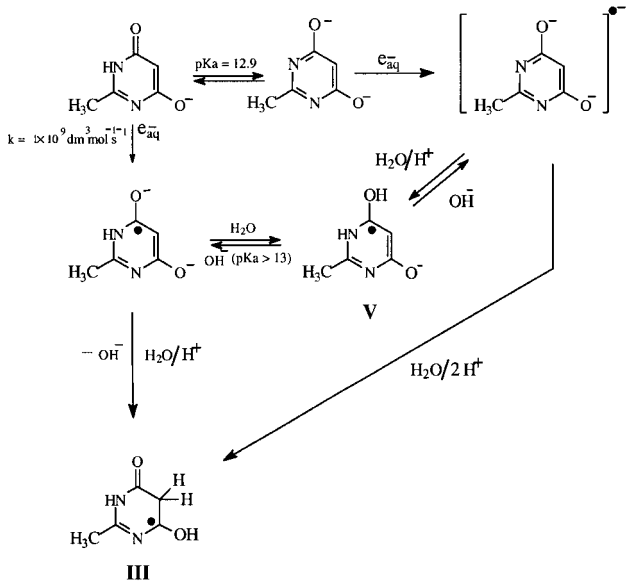
$$EA + MV^{2+} \xrightarrow{k_2} MV^{\cdot+} \tag{4}$$

$$EA \xrightarrow{k_t} EA' \tag{5}$$

where k_2 is the second-order rate constant, k_t is the rate of transformation and EA′ represents the transformed electron adducts.

$$G = 2.7 \times \frac{k_2[MV^{2+}]}{k_2[MV^{2+}] + k_t} \qquad \text{(eqn.1)}$$

where 2.7 is the total yield of the electron adduct. According to this calculation, the expected G value of $MV^{\cdot+}$ is 2.35. However, the observed $G(MV^{\cdot+})$ is 2.7 (Table 2). This indicates that the transformed species is also reducing in nature with respect to MV^{2+}. In order to confirm further the nature of the transformed electron adduct, $G(MV^{\cdot+})$ was determined in the presence of 0.3 mol dm^{-3} phosphate at pH 7.5. The observed G value was 2.7. Based on these observations, it is proposed that the transformed species is a C(5)-protonated C(6)-yl form of DHMP (**III**). The structure **III** is in resonance with **IV**.



Scheme 3

Both **III** and **IV** can be reducing in nature with respect to MV^{2+}. The ketonic form of structure **IV** (as shown in Scheme 2), reported in the case of dihydroxypyrimidine,[18] is also probable. However, the speed of this enol–keto transformation could not be determined. It is interesting that the phosphate-catalysed carbon protonation in the case of uracil and its methyl derivatives leads to a non-reducing C(5)-yl radical, whereas in the present case it is a C(6)-yl radical. Additional support for the formation of **III** is its spectral similarity with H adducts. (see later for further explanation). However, a simultaneous formation of a possible C(5)-yl radical (non-reducing), as was reported with other pyrimidines,[16,17] can be completely ruled out at pH 4.5 based on the $G(MV^{\cdot+})$ value. Furthermore, it is important to note that in the reported ESR study with dihydroxypyrimidine systems, at pH 4–5 the observed ESR spectrum corresponds to H adducts.[18] This report is clearly in line with our observation on DHMP that in the initial stage, an oxygen-protonated electron adduct is formed which is later transformed in the presence of H^+ to a C(5)-protonated electron adduct which is similar to the H adduct. The difficulties in the detection of an oxygen-protonated electron adduct in the previous study[18] may be due to the longer time-scales that are normally used in ESR experiments, on which the C(5) protonation must be completed.

At pH 9, DHMP exists predominantly in its deprotonated form ($pK_a = 6.35$). Therefore, the reaction of e_{aq}^- at this pH must be considered as the addition of e_{aq}^- to the monoanion of DHMP [the decrease in the second-order rate constant at pH 9 is a clear indication of this reaction (Table 1)]. Such an addition reaction would definitely lead to the formation of a radical dianion as shown in Scheme 3. The radical dianion can be protonated by water so that a radical monoanion can result. We propose that the species with absorption maxima at 310 and 360 nm at pH 9 is the monoanionic form of the electron adduct of DHMP (**V**) as represented in Scheme 3. The supporting evidence for this argument came from ESR and conductance studies on dihydroxypyrimidine systems between pH 6 and 12.[18] The conductance measurements at pH 8.5–9.5 yielded the quantitative production of OH^-, which clearly supported the protonation reaction.[18] Furthermore, the ESR study indicated the formation of a ketyl-type radical monoanion whose unpaired spin is strongly delocalized.[18] Our results with MV^{2+} gives a clear indication of the reducing property of the radical anion (**V**) at pH 9. At this pH the observed $G(MV^{\cdot+})$ value (2.7) represents the full yield of electron adduct (Table 2). Therefore, it is expected that the unpaired spin in DHMP radical anion (**V**) is more localized towards the ketyl carbon, as reported in other similar pyrimidine electron adduct studies.[16,17] As can be seen from Fig. 2, the spectral nature of the intermediate at pH 13 is similar to that at pH 9, indicating the similarity of the species existing at these two pHs.

Since DHMP has its second pK_a at 12.9, the reaction of e_{aq}^- at pH 13 is with a mixture of about 56% dianionic form and 44% monoanionic form of DHMP. The attack at the dianionic form would give rise to a radical trianion in the initial stage which could be immediately protonated by water. Since the spectral features at pH 9 and 13 are very similar, it is logical to assume that the trianion radical is protonated twice to form a monoanion radical of DHMP with a structure similar to that obtained at pH 9 (Scheme 3). The similar spectra obtained both at pH 9 and 13 may be an indication of a much higher pK_a value (>13) for the monoanion radical of DHMP. The ESR study on dihydroxypyrimidine systems reported the presence of a radical dianion protonated at C(5) (catalysed by OH^-) and its subsequent protonation leads to a neutral radical similar to the H adduct.[18] The C(5)-protonated dianion could be detected only at $[OH^-] \geq 0.2\ mol\ dm^{-3}$.[18] However, the percentage distribution of the radical monoanion and the neutral H adduct of DHMP (existing at pH >9, if formed) could not be obtained using MV^{2+} in the present case as both the structures can be reducing in nature with respect to MV^{2+}. The quantitative yield of $MV^{\cdot+}$ at pH 13 when the $G(e_{aq}^-)$ is 3.3 due to the conversion of $H^\cdot$ to e_{aq}^- by OH^-, reveals once again the facts that the electron adducts are reducing in nature and that the unpaired spin is located at C(2), C(4) or C(6) positions. Furthermore, the protonation at C(6) leading to the formation of a C(5)-yl radical of DHMP, as observed with uracil and thymine,[16] is negligible in the case of DHMP. The quantitative yield of $MV^{\cdot+}$ is in line with such a conclusion.

2,4-Dimethyl-6-hydroxypyrimidine (DMHP). The electron adduct spectrum showed a single distinct absorption

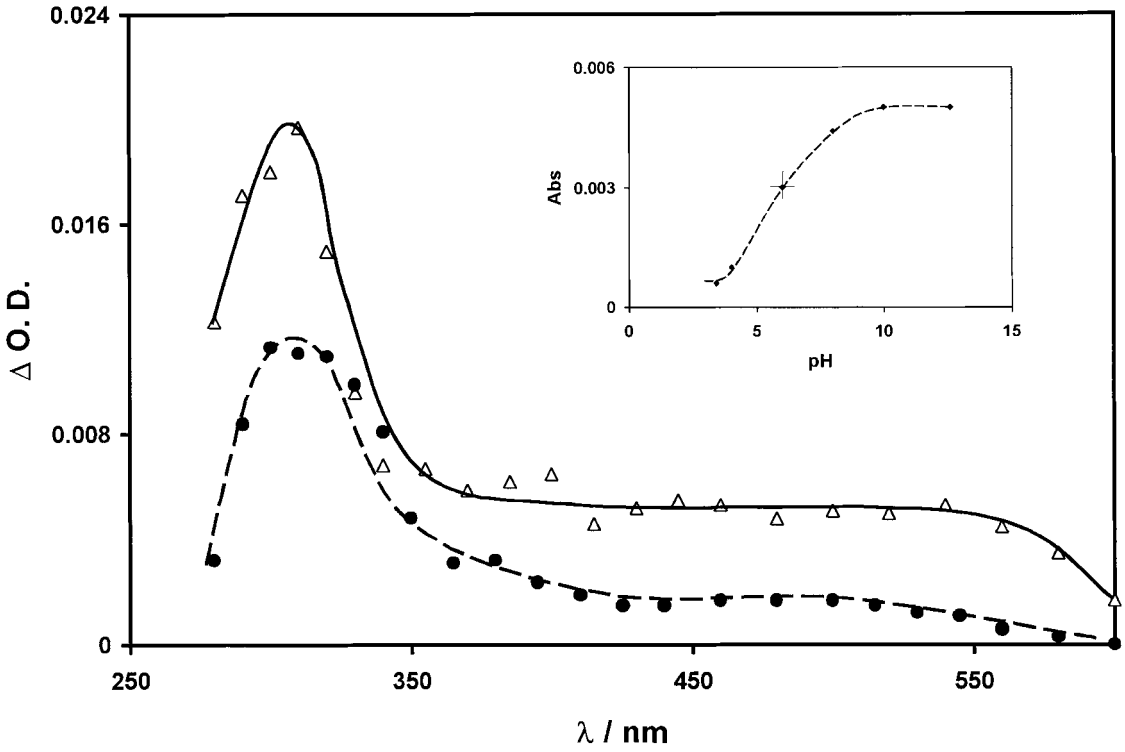
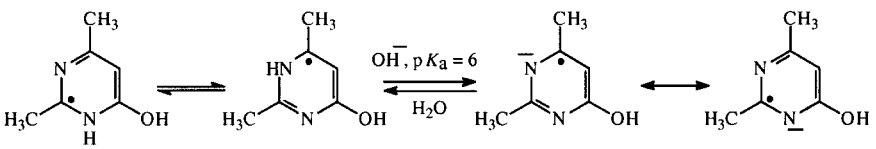


Figure 3. Transient absorption spectra obtained in N_2-saturated aqueous solutions of DMHP (1×10^{-3} mol dm^{-3}) containing propan-2-ol (0.2 mol dm^{-3}) at pH 12.5 (△) and 4.5 (●) at 2 μs after the pulse; dose per pulse ≈ 15 Gy. Inset: dependence of absorbance of the intermediate on pH at 430 nm. The inflection point is at pH 6

maximum at 310 nm at pH 4.5 (Fig. 3) and at pH 6 (not shown) and were very similar with a slight difference in the absorbance values. The time-resolved spectra at both pH values did not show any indication of a transformation reaction as was observed with DHMP, but a second-order decay. The nature of the spectrum at pH 12 was also similar but the absorbance at the λ_{max} was significantly higher (Fig. 3). A plot of absorbance versus pH of the electron adduct (Fig. 3, inset) at 430 nm showed an inflection point around pH 6.0. The spectrum obtained in presence of 0.3 mol dm^{-3} phosphate did not show much difference from that obtained without phosphate, indicating the absence or a relatively slow transformation reaction on the pulse radiolysis scale. A nearly quantitative yield of $MV^{\cdot+}$ (>90%) at pH 6 and 12.5 clearly indicated the reducing nature of the electron adduct (see Table 1).

From the spectral behaviour of the electron adduct of DMHP at pH 4.0, 6.0 and 12.5 and from the protonation/deprotonation equilibrium with a p*K* value of 6.0 (Fig. 3), it can be assumed that at low pH the electron adduct is protonated. Therefore, the species existing at higher pH such as at pH 12.5 (the spectrum is identified with its λ_{max} at 310 nm) is the deprotonated form of the electron adduct. A similar behaviour of the electron adduct of uracil and 1,3-dimethylthymine has already been reported.[16,17] In aqueous medium DMHP is likely to be in its enolic form [In a recent study,[26] we showed that both DMU and MU undergo rapid deprotonation at near neutral pH, whereas DHMP and DMHP did not show such a phenomenon. This is observed only with pyrimidines containing H at N(1) and is explained on the basis of the deprotonation of the N(1)H. Therefore, this is additional support for the conclusion that DMHP exists in its enolic form at C(6).] Therefore, the expected sites of attack are at N(1) or N(3) and the resulting radical anion can be easily protonated by H_2O/H^+ [reaction (6)].

Although DMHP is in its deprotonated form at pH 12.5, the radical dianion resulting from the e_{aq}^- attack is likely to be protonated by water, similarly to the dianion of DHMP, leading to the radical monoanion which undergoes protonation only at lower pH. Both protonated and deprotonated forms can be reducing in nature with respect to MV^{2+} since the unpaired spin density is at



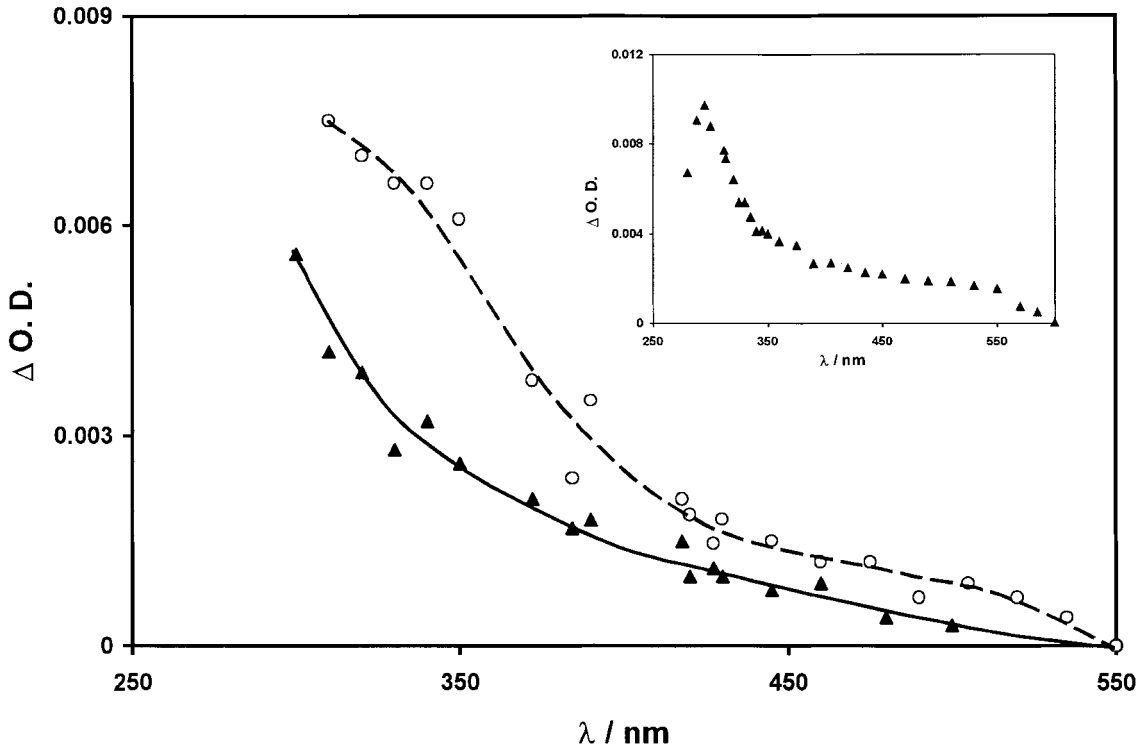
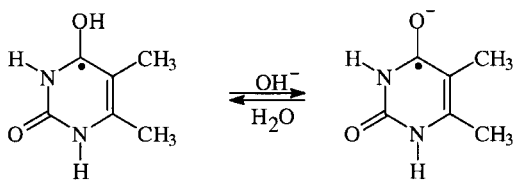


Figure 4. Transient absorption spectra recorded in N_2-saturated aqueous solutions of DMU (1×10^{-3} mol dm^{-3}) containing propan-2-ol (0.2 mol dm^{-3}) at pH 12 (○) and pH 6 (▲) at 30 μs after the pulse; dose per pulse ≈ 16.7 Gy. Inset: transient absorption spectra obtained with MU (1×10^{-3} mol dm^{-3}) at pH 6 (2 μs after the pulse) under similar conditions; dose per pulse ≈ 14.5 Gy

carbon. The high $G(MV^{\cdot+})$ values at lower and higher pHs reflect this reducing property.

5,6-Dimethyluracil (DMU) and 6-methyluracil (MU). The electron adduct spectra of DMU and MU were recorded in the presence of 0.01 mol dm^{-3} phosphate buffer in order to minimize the possible formation of DMU anion resulting from the reaction of radiolytically produced OH^- and DMU (see above[26]). The DMU and MU anions are reported to have λ_{max} at 295 and 285 nm, respectively.[26] The transient spectra at pH 6 and 12 showed a single λ_{max} around 300 nm but there was a clear difference in their absorbance values (Fig. 4). The observed $G(MV^{\cdot+})$ at pH 6.0 was 2.7, which corresponds to the total yield of the electron adducts, and a similar yield of 2.8 was also calculated at pH 12.0. Hence the electron adduct of DMU also appears to have similar properties to those in the case of DHMP.

The protonated electron adduct and its deprotonated forms can be distinguished from the difference in their absorption coefficients at the λ_{max} (Fig. 4). The preferred site of attack is proposed to be at O(4) and O(2). It is reported in the case of uracil that the O(2)-protonated electron adduct has an absorption maximum less than 280 nm at pH 5.1.[25] Therefore, it can be concluded that the species with λ_{max} at 300 nm is the O(4)-protonated electron adduct of DMU which is in its deprotonated form at higher pH as shown in reaction (7)



(7)

The high yields of $MV^{\cdot+}$ at pH 6 and 12.5 (see Table 2) are in agreement with the above explanation. Both protonated and the deprotonated forms can be reducing with respect to MV^{2+}. In this case too the phosphate-induced protonation at carbon [C(5) and C(6)] is relatively slow and this was confirmed from the absence of any spectral changes between 380 and 520 nm in the presence of 0.3 mol dm^{-3} phosphate. The transient spectrum obtained with MU is also similar to the spectrum of DMU and hence a similar electron adduct protonated at O(4) is proposed at pH 6. The spectrum showed a single λ_{max} at 295 nm. This is in agreement with the reported electron adduct spectrum of MU.[25] The $G(MV^{\cdot+})$ values at pH 6 (i.e. 2.6) and at pH 12 (i.e. 3.3) clearly indicate the quantitative formation of the reducing radical, the electron adduct protonated at O(4) at pH 6 and its deprotonated form at pH 12, which is in line with the earlier report.[25]

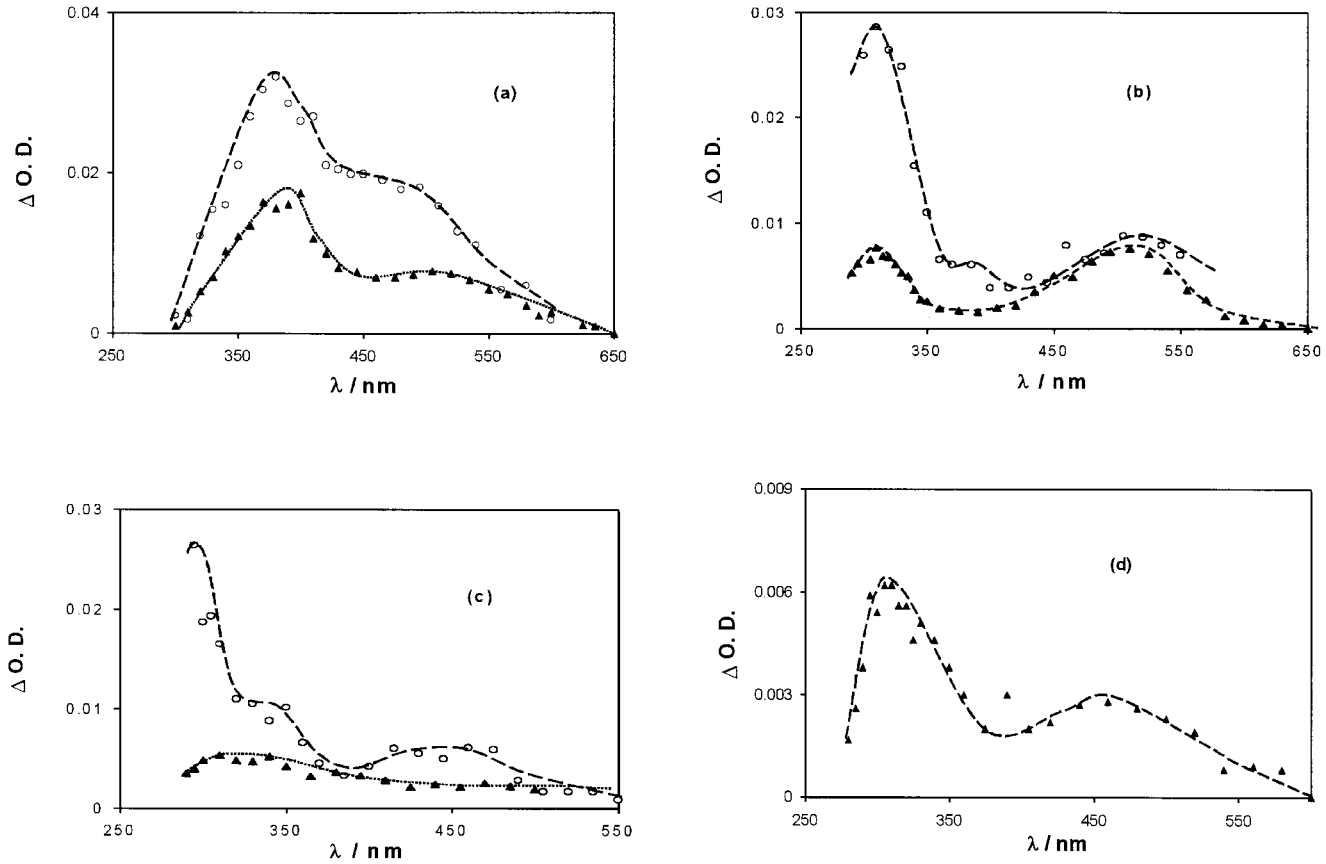


Figure 5. H adduct spectra recorded at 2 μs after the pulse of (a) DHMP at pH 4.5 (○) and at pH 1 (▲); (b) DMHP at pH 6 (○) and pH 1 (▲); (c) DMU at pH 6 (○) and 1 (▲); and (d) MU at pH 1 (▲). All experiments at near neutral pH were carried out in N_2O-saturated aqueous solutions and that at pH 1 was in N_2-saturated aqueous solutions containing typically 1×10^{-3} mol dm^{-3} substrate at dose per pulse ≈ 16 Gy. The ΔO.D.s are calculated for a typical *G*(H) value of 3.3

Reactions of H atom: kinetics and spectra

The reactions of H˙ with DHMP, DMHP, DMU and MU were studied at pH 1 and at near neutral pH. The reaction at neutral pH was studied in N_2O-saturated solutions containing 2-methylpropan-2-ol to scavenge ˙OH. The second-order rate constants were determined from the rate of build-up at the respective absorption maxima of the H adduct. These rate constants were found to be in the range (1.7–28) $\times 10^8$ dm^3 mol^{-1} s^{-1} at pH 1 and (9.2–26) $\times 10^8$ dm^3 mol^{-1} s^{-1} at near neutral pH (for individual rate constants, see the supplementary material). It is evident that the pK_a values of the substrates have a profound effect on the rate of reaction. The bimolecular rate constants at pH 1 for all the pyrimidines were considerably lower than those at higher pH except in the case of DHMP, where the values at pH 1 and 4.5 were comparable. The comparatively low rate constants at lower pH values are understandable as H attack is electrophilic. Since DHMP has a pK_a value of only 0.21, the rate constant value remained unaffected.

The absorption spectra obtained for the reaction of ˙H with DHMP, DMHP, DMU and MU are given in Fig. 5(a–d) (the spectral parameters such as the absorption maxima and the absorption coefficients at the respective maximum are available in the supplementary material). The H adduct spectra of all these compounds were found to undergo a second-order decay at their absorption maxima.

The preferred sites of attack of H˙ in uracil systems are the C(5) and C(6) positions.[9–11] Therefore, similar sites of attack are likely in the case of DHMP. It is therefore assumed that C(6)-yl radical is formed from the attack of H˙ at the C(5) position of DHMP as H˙ is an electrophile and therefore it can attack at the more electron-rich C(5) position (Scheme 2). The difference in the absorbance at lower and higher pHs as can be seen in Fig. 5 must be due to the protonation of the parent pyrimidine. This difference is more prominent with DMHP at pH 1.0 where it is fully in its protonated form. At this point, it is interesting to compare the transformed electron adduct spectrum at pH 4.5 and the spectrum at pH 7.5 in presence of phosphate (Fig. 1) with the H adduct spectrum at pH 4.5 in the case of DHMP [Fig. 5(a)]. It can be seen that these three spectra are similar and hence it is logical to assume that the intermediate species are also similar.

Table 2. Products identified from the reaction of e_{aq}^- with DHMP by HPLC–ES-MS analysis (eluent; water; flow-rate; 0.5 dm^3 min^{-1})

Product[a]	Molecular formula	Molecular weight	*m/z*
(**a**) 5,5-Dihydro-6-hydroxy-2-methyl-(3*H*)-pyrimidin-4-one	$C_5N_2O_2H_8$	128	+129
(**b**) 6-Hydroxy-2-methyl-(3*H*)-pyrimidin-4-one	$C_5N_2O_2H_6$	126	−125, +127
(**c**) 5,5-Dihydro-2-methyl-(3*H*)-pyrimidin-4,6-dione	$C_5N_2O_2H_6$	126	+126
(**d**) Bis[5,5-dihydro-6-hydroxy-2-methyl-(3*H*)-pyrimidin-4-one-6-yl]	$C_{10}N_4O_4H_{14}$	254	−253
(**e**) 2-Methyl-(3*H*)-pyrimidin-4-one-6-[5,5-dihydro-6-hydroxy-2-methyl-(3H)-pyrimidin-4-one]	$C_{10}N_4O_3H_{12}$	236	+236
(**f**) Bis[2-methyl-(3*H*)-pyrimidin-4-one-6-yl]	$C_{10}N_4O_2H_{10}$	218	−217

[a] Retention times of the products: (**a**) 1.1, (**b**) 0.95, (**c**) 2.5, (**d**) and (**e**) 1.2 and (**f**) 2.3 min.

Similarly to DHMP, formation of a C(5)HC(6)-yl radical is proposed in the case of DMHP which exists in its deprotonated form at pH 6. However, in the case of DMU and MU, both C(6)HC(5)-yl and C(5)HC(6)-yl radicals are proposed to be formed. Based on the preferential formation of C(6)HC(5)-yl radical in the case of thymine,[18] it can be expected that a similar trend is likely in DMU and MU. It can be further seen from the H adduct spectrum of DMU (Fig. 5) that at $\lambda < 300$ nm there is a disproportionate difference between the spectra at pH 6 and pH 1. This difference is mainly due to the interference of the DMU anion spectrum which has a λ_{max} at 295 nm under N_2O-saturated conditions (pH 6) as discussed earlier.[26]

Product analysis

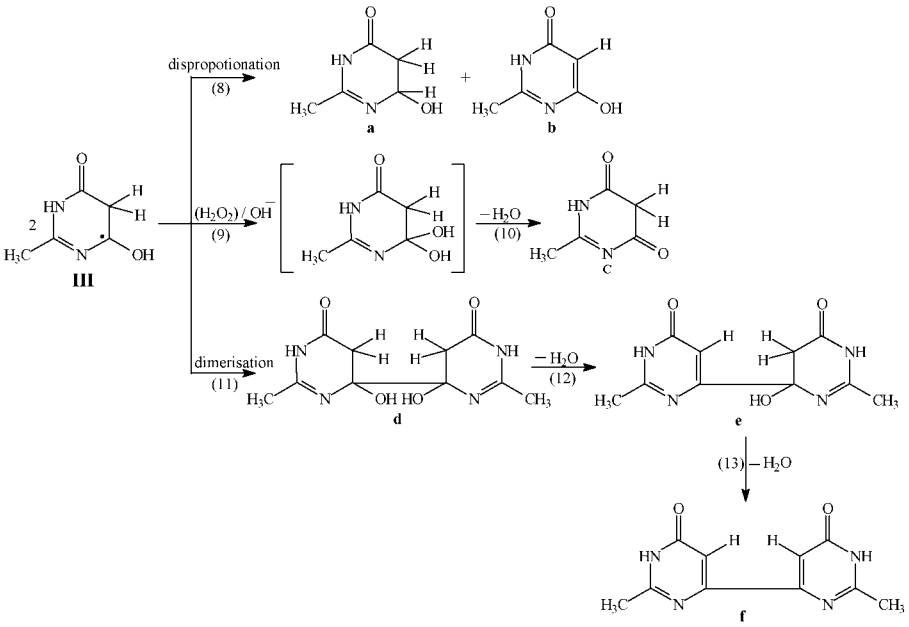
Qualitative product analysis was carried out after prolonged γ-radiolysis (dose $\approx$ 2000 Gy) of argon-saturated solutions containing either DHMP or DMHP (1×10^{-3} mol dm^{-3}) in presence of 2-methylpropan-2-ol (0.2 mol dm^{-3}) using HPLC–ES-MS. Even with the prolonged irradiation it was observed that only a minor portion of the substrate has been consumed and the products peaks were too feeble (representative HPLC results and mass spectra obtained in the case of DMHP are available in the supplementary material). A series of *m/z* values were obtained from these feeble product peaks. However, from the mass peaks, the most probable *m/z* values were selected for the most probable products. The identified products, their molecular weights and the *m/z* values obtained from the MS data are summarized in Tables 2 and 3, for DHMP and DMHP, respectively.

From the identified products, it is almost clear that these products are the result of a bimolecular reaction of the protonated electron adducts **III** [C(5)-protonated C(6)-yl form of DHMP] and **VII**. The structure **VII** [N(1)-protonated C(6)-yl form of DMHP] is the mesomeric form of the protonated electron adduct of DMHP [see reaction (6) and Scheme (5)]. The structures of these electron adducts were demonstrated with the help of MV^{2+} using the pulse radiolysis experiments (see earlier). Furthermore, these products are proposed to result from mainly disproportionation and dimerization reactions as shown in Schemes 4 and 5. In the case of DHMP at pH 4.5, the disproportionation reaction leads to the products **a** and **b** [Scheme 4, reaction (8)] where **b** is the starting compound. Indication is obtained for the formation of a product like **c**, which could be the result of an unstable intermediate C(6)-dihydroxy product as shown in reaction (9). This can be resulted from the oxidation of **III** followed by addition of OH^-. Such an oxidation is possible either by the disprotonation reaction

Table 3. Products identified from the reaction of e_{aq}^- with DMHP by HPLC–ES-MS analysis (eluent; water; flow-rate; 0.5 dm^3 min^{-1})

Product[a]	Molecular formula	Molecular weight	*m/z*
(**g**) 5,5-Dihydro-6-hydroxy-2,4-dimethylpyrimidine	$C_6N_2OH_{10}$	126	−125, +127
(**h**) 5-Hydro-6-hydroxy-2,4-dimethylpyrimidine	$C_6N_2OH_8$	124	−123, +125
(**i**) Bis(5,5-dihydro-6-hydroxy-2,4-dimethyl pyrimidin-6-yl)	$C_{12}N_4O_2H_{18}$	250	n.d.[b]
(**j**) 2,4-Dimethyl-6-(5,5-dihydro-6-hydroxy-2,4-dimethylpyrimidin-6-yl)	$C_{12}N_4OH_{16}$	232	−232
(**k**) Bis(2,4-dimethylpyrimidin-6-yl)	$C_{12}N_4H_{14}$	214	+214
(**l**) 2,4-Dimethylpyrimidin-6-one	$C_6N_2OH_8$	124	−123, +125

[a] Retention times of the products: (**g**) 8.5, (**h**) and (**l**) 5.3, (**j**) 5.6 and (**k**) 5.7 min.
[b] Not detected.

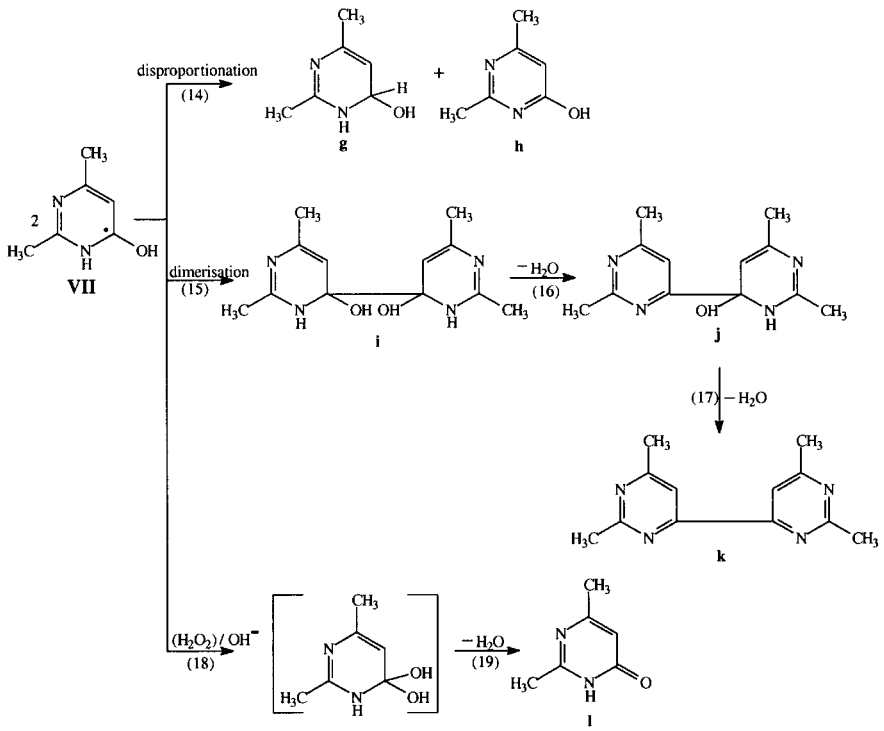


Scheme 4

of **III** or by the reaction of radiolytically formed H_2O_2. The formation of dimers is another important reaction pathway which can lead to products with the structures **d, e** and **f**. Structures **e** and **f** are formed via stepwise elimination of water forming the initial dimer as shown in Scheme 4.

As there is very little information available about the product formation from the reaction of e_{aq}^- with pyrimidine, no fruitful comparison of these products could be made. However, a stepwise elimination of water from the dimeric products of pyrimidine is reported using conventional chromatographic technique.[27]

DMHP also gave similar product pattern like DHMP at pH 6 (Scheme 5). The products **g** and **h** (where **h** is the starting compound, DMHP) are the result of a disproportionation reaction. The *m/z* value corresponding to the



Scheme 5

dimer **i** is, however, not seen in the MS data. On the other hand, the products **j** and **k** resulting from the successive water elimination from the dimeric product **i** were seen (Table 3). The only possible reason for its absence is probably its instability leading to fast water elimination [reactions (16) and (17)]. The product **l** is only the keto form of DMHP (i.e. **h**) and it is included in the scheme to demonstrate the possibility of an alternative pathway for the regeneration of the DMHP which normally exists in its enol form (see earlier[26])

CONCLUSION

The preferential protonation at the C(5) position of the electron adduct of DHMP by H^+ and phosphate was demonstrated from its spectral features and with the help of its reaction with the oxidant methylviologen using a pulse radiolysis technique. A similar species is formed on $H^{\cdot}$ attack at the C(5) position of DHMP. The formation of such C(5)-protonated C(6)-yl radical is only a minor process with other pyrimidine derivatives. The electron transfer from a carbon-protonated electron adduct of thymine to molecular oxygen or nitro aromatic radiosensitizers is a restricted process.[17] The present study demonstrates that the nature of the substituents at the pyrimidine ring has a profound effect on the redox properties of the electron adducts as the C-protonated electron adduct of DHMP transfers an electron to radiosensitizers such as MV^{2+}. The C(5) and C(6) protonation of the electron adducts of DMHP, DMU and MU by phosphate is found to be a very slow process, at least on the pulse radiolysis scale ($k < 10^4\ s^{-1}$). Product analysis from the reaction of e_{aq}^- with pyrimidine has been a relatively difficult area and this study qualitatively identified a number of important products resulting from mainly disproportionation and dimerization of the protonated electron adducts of DHMP and DMHP.

Acknowledgements

Financial support from the Board of Research in Nuclear Sciences (BRNS), Government of India, is gratefully acknowledged. T.A.J. and V.M.M. are grateful to the BRNS and the Nuclear Science Centre (NSC), respectively, for Junior Research Fellowships. T.A.J. is also thankful to the CSIR for a Senior Research Fellowship. We thank the Rubber Research Institute of India (RRII), Kottayam, for γ-radiolysis experiments.

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