# The $SO_4^{\cdot-}$-induced Oxidation of 2′-Deoxyuridine-5′-phosphate, Uridine-5′-phosphate and Thymidine-5′-phosphate. An ESR Study in Aqueous Solution

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*In memoriam Professor Dr. O. E. Polansky*

Free Radicals, ESR, Pyrimidine-5′-nucleotides, Radical Cation

Reactions of photolytically generated $SO_4^{\cdot-}$ with 2′-deoxyuridine-5′-phosphate (5′-dUMP), uridine-5′-phosphate (5′-UMP) and thymidine-5′-phosphate (5′-dTMP) were studied by ESR spectroscopy in aqueous solution under anoxic conditions. From 5′-dUMP and 5′-UMP the 5′,5-cyclic phosphate-6-yl radicals **10** and **11** were generated (pH 2–11) whereas from 5′-dTMP at pH 3–8 the 5,6-dihydro-6-hydroxy-5-yl radical **14** and at pH 7–11 the 5-methylene-2′-deoxyuridine-5′-phosphate radical **15** was produced. In the experiments with 5′-UMP in addition to radical **11** the signals of sugar radicals **12** and **13** were detected. It is assumed that the base radical cations act as intermediates in the $SO_4^{\cdot-}$-induced radical reactions. The 5′-phosphate group adds intramolecularly to the C(5)–C(6) bond of the uraclilyl radical cation whereas the thymidyl radical cation of 5′-dTMP reacts with $H_2O$ at pH $< 8$ to yield the 6-OH-5-yl adduct **14** and deprotonates at pH $> 7$ thus forming the allyl-type radical **15.** In 5′-UMP transfer of the radical site from the base to the sugar moiety competes with intramolecular phosphate addition.

## Introduction

The damaging effect of ionizing radiation on nucleic acids can be described by two mechanisms known as the "direct" and the "indirect" effect [1]. The main damaging species in the "indirect effect" is the OH radical formed by radiolysis of $H_2O$ whereas by the "direct effect" solvated electrons, radical cations and radical anions, especially of the nucleobases, are formed in the primary step. The conditions of the "direct effect" are met by γ-irradiating substrates in the solid state [2] or in frozen aqueous solution [3]. Investigation of the "direct" effect in fluid aqueous solution is hampered by the fact that the absorption of ionizing radiation is always accompanied by the formation of the intermediates of the "indirect" effect. One way to circumvent these difficulties is to ionize with UV light [4]. On the other hand, it should be possible to use radiomimetic agents, like *e.g.* $SO_4^{\cdot-}$, to generate radical cations of the nucleobases and thus to induce radical reactions which occur upon the "direct" damage of DNA. Recent studies [5–7] have shown that from N(1)-methylated pyrimidines [5] like *e.g.* 1-methyluracil (1-MeU) and 1-methylthymine (1-MeT), and from the 2′-deoxyribose derivatives [6, 7] 2′-deoxyuridine (dU) and thymidine (dT), by reaction with $SO_4^{\cdot-}$ the ESR spectra of the OH adduct radicals **1, 2, 5, 6** were generated whereas in the presence of phosphate dianions, the phosphate adduct radicals **3** and **4** of 1-MeU and 1-MeT were detected. In the experiments with pyrimidine-ribose derivatives sugar radicals were observed [6, 7] like *e.g.* species **7** and **8** derived from uridine. The spectral parameters of radicals **1–8** are given in Table I for comparison.

In the present paper, reactions of $SO_4^{\cdot-}$ with 2′-deoxyuridine-5′-phosphate (5′-dUMP), uridine-5′-phosphate (5′-UMP) and thymidine-5′-phosphate (5′-dTMP) are investigated in aqueous solution at 277 K. The radicals detected in the experiments with the 5′-nucleotides were quite different from those observed earlier in the corresponding experiments with the N(1)-methylated nucleobases [5], the 2′-deoxyribonucleosides (dT and dU) [6], and with thymidine-3′-phosphate (3′-dTMP) (this work).

R=H, X=H, Y=OH, Z=$OPO_3^{2-}$: 5′-d UMP

R=H, X=OH, Y=OH, Z=$OPO_3^{2-}$: 5′-UMP

R=$CH_3$, X=H, Y=OH, Z=$OPO_3^{2-}$: 5′-d T MP

R=$CH_3$, X=H, Y=$OPO_3^{2-}$, Z=OH: 3′-d T MP

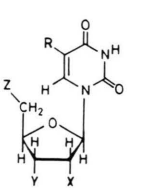

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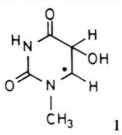
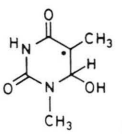
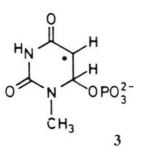
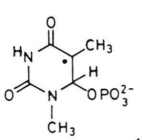
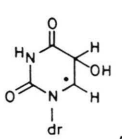
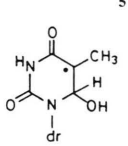
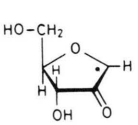
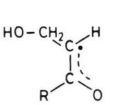
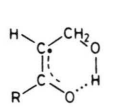
Table I. ESR parameters of the radicals generated from 1-MeT, 1-MeU, dT, dU, and uridine with $SO_4^{\cdot-}$.

| Substrate | Radical | pH | Hyperfine splittings (mT)[a] a(H-α) | a(H-β) | a(other) | $g^b$ |
|---|---|---|---|---|---|---|
| 1-MeU[c] |  1 | 2–9 | 1.83 (6-H) | 2.04 (5-H) | 0.09 (N)<br>0.048 (N)<br>0.09 (1-$CH_3$) | 2.0028 |
| 1-MeT[c] |  2 | 3–7 | | 1.51 (6-H)<br>2.26 (5-$CH_3$) | 0.058 (N)<br>0.011 (N)<br>0.150 ($N^3$–H)<br>0.037 (6-OH) | 2.0031 |
| 1-MeU[c] |  3 | 6.5–9.5 | 2.05 (5-H) | 1.56 (6-H) | 0.051 (N)<br>0.025 (N)<br>0.138 ($N^3$–H)<br>0.13 ($^{31}$P) | 2.0032 |
| 1-MeT[c] |  4 | 6.5–9.5 | | 1.18 (6-H)<br>2.29 (5-$CH_3$) | 0.045 (N)<br>0.045 (N)<br>0.14 ($N^3$–H)<br>0.038 ($^{31}$P) | 2.0032 |
| dU[d] |  5 | 2–9 | 1.88 (6-H) | 2.20 (5-H) | 0.28 (1′-H) | 2.0028 |
| dT[d,e] |  6 | 3–7 | | 1.125 (6-H)<br>2.23 (5-$CH_3$) | [f] | 2.0032 |
| Uridine[e] |  7 | 2–9 | 1.36 (1′-H) | 0.54 (3′-H) | 0.25 (4′-H) | 2.0049 |
| Uridine[e,g] |  8a | 7–11 | 1.87 (4′-H) | 2.62 (5′-$CH_2$) | 0.13 (2′-H) | 2.0043 |
| Uridine[e,g] |  8b | 7–11 | 1.81 (4′-H) | 2.83 (5′-$CH_2$) | 0.14 (2′-H) | 2.0045 |

[a] ± 0.005 mT; [b] ± 0.0001; [c] data from ref. [5]; [d] dr = 2′-deoxy-1-ribosyl; [e] data from ref. [6]; [f] small couplings not resolved; [g] R = uracil–CH(OH)–CH(OH)–.

## Results

*ESR spectroscopy*

5′-dUMP

The spectrum obtained upon reaction of $SO_4^{\cdot-}$ with 5′-dUMP (Fig. 1) consisted of four groups of signals ($g$ = 2.0028). The pattern is consistent with two large proton couplings of similar values (1.89 and 1.65 mT at pH 7). From the splittings of the individual groups of lines four small coupling constants were evaluated which did not change upon deuteration with $D_2O$ [a(H-1′), a($^{31}$P) and two nitrogen splittings]. The values of all splittings decreased at pH ~9, *i.e.* in the region expected for deprotonation at N(3). (A value for $pK_a$ of 10.2 was determined for the 5-OH adduct radical [8] of 5′-dUMP by pulse radiolysis with optical detection [9]). By comparison with the rather similar spectrum of radical **1** derived from 1-MeU one might conclude, at first glance, that we deal with the 5-OH adduct of 5′-dUMP. This assignment was ruled out, however, by comparison with a series of OH adduct radicals derived from uracil nucleosides and nucleotides upon addition of OH radicals to the base moiety (radicals **5** and **9a–9e,** Table II). It is known from pulse radiolysis studies that under those conditions predominantly the 5-OH-6-yl radicals are generated [10]. In agreement with this expectation, the spectra were characterized by two large doublets and a small one due to the α- and β-proton and 1′-H of the sugar residue [11]. Deprotonation of N(3) at pH ~10 resulted in a decrease of the sum a(H-α) + a(H-β) by ~0.5 mT. The $g$ factor of radicals **5** and **9a–9e** (Table II) was 2.0028 which is characteristic for uracil-6-yl radicals whereas for the isomeric 6-OH-5-yl radicals slightly higher values for $g$ ($g$ ~ 2.0033) are expected due to delocalization of the unpaired electron to oxygen(4) [5, 12]. It turned out that the two large proton couplings of the 5-OH adducts **5** and **9a–9e** were only slightly different for nucleosides and nucleotides and rather insensitive to the position of the phosphate group in the nucleotides. They were, however, significantly larger than the corresponding couplings in the spectrum derived with $SO_4^{\cdot-}$ from 5′-dUMP. Final assignment of the rather unusual spectra in Fig. 1 takes into consideration the fact that in nucleosides and nucleotides the base rings exist in two broad bands of conformations, labeled *anti* and *syn* depending on whether or not O(2) projects

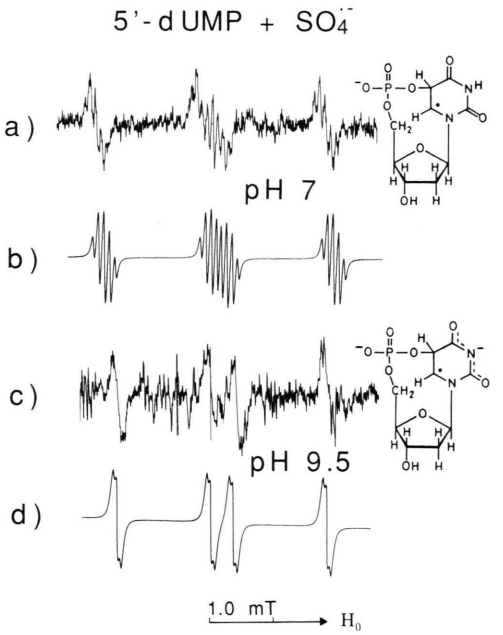


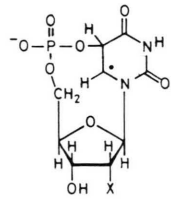
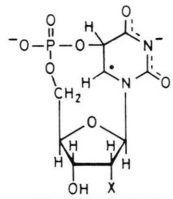
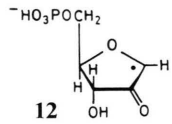
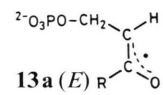
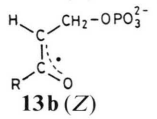
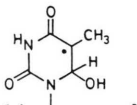
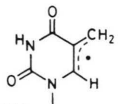
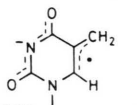
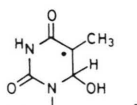

Fig. 1. ESR spectra obtained upon *in situ* photolysis of a solution containing 5′-dUMP ($10^{-3}$ mol × $l^{-1}$), $K_2S_2O_8$ ($5 \times 10^{-3}$ mol × $l^{-1}$) and acetone (1%); a) pH 7; c) pH 9.5; b) and d) simulations with the parameters given in Table III for radicals **10a** and **10b**.

Table II. Hyperfine splittings of the 5-OH-6-yl radicals **5** and **9a–9e** of uracil nucleosides and nucleotides, pH 7. The OH radicals were generated either by photolysis of $H_2O_2$ (pH 2–8) or by radiolysis of $N_2O$-saturated solutions (pH 2–12). At pH ~10 the sum a(H-α) + a(H-β) decreases by ~0.5 mT. The $g$ factor of radicals **5** and **9a–9e** is 2.0028.

| Substrate | Radical | Hyperfine splittings (mT)[a] a(H-α) | a(H-β) | a(H-1′) |
|---|---|---|---|---|
| 5′-dUMP[b] | **9a** | 1.88 | 1.88 | 0.3 |
| 5′-UMP[b] | **9b** | 1.91 | 1.91 | 0.3 |
| 3′-UMP[c] | **9c** | 1.88 | 2.18 | 0.3 |
| poly(U)[c] | **9d** | 1.85 | 2.15 | 0.33 |
| Uridine[c] | **9e** | 1.85 | 2.15 | 0.3 |
| dU[c] | **5**[d] | 1.88 | 2.20 | 0.28 |

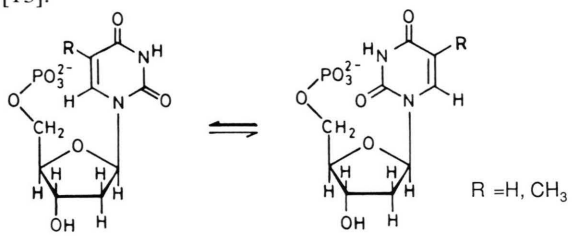
[a] ± 0.01 mT; [b] this work; [c] data from ref. [7]; [d] radical **5** is generated with $SO_4^{\cdot-}$ and with ·OH.

Table III. Radicals derived from 5′-dUMP, 5′-dTMP and 3′-dTMP upon reaction with $SO_4^{\cdot-}$.

| Substrate | Radical | pH | Hyperfine splittings (mT)[a] a(H-α) | a(H-β) | a(other) | $g$[b] |
|---|---|---|---|---|---|---|
| 5′-dUMP (X = H) 5′-UMP (X = OH) |  X = H: **10a** X = OH: **11a** | 2–9 | 1.89 (6-H) | 1.65 (5-H) | 0.08 (1′-H) 0.08 ($^{31}$P) 0.09 (N) 0.01 (N) | 2.0028 |
| |  X = H: **10b** X = OH: **11b** | 9–11 | 1.80 (6-H) | 1.45 (5-H) | 0.04 (1′-H) 0.06 ($^{31}$P) 0.02 (N) 0.02 (N) | 2.0028 |
| 5′-UMP |  **12** | 2–8 | 1.36 (1′-H) | – | 0.54 (γ-H) 0.25 (γ-H) | 2.0049 |
| 5′-UMP[c] |  **13a** (*E*) | 7–11 | 1.85 (4′-H) | 2.77 (5′-$CH_2$) | 0.16 (2′-H) 0.06 ($^{31}$P) | 2.0043 |
| |  **13b** (*Z*) | 7–11 | 1.87 (4′-H) | 2.86 (5′-$CH_2$) | 0.14 (2′-H) 0.06 ($^{31}$P) | 2.0045 |
| 5′-dTMP[d] |  **14** dr-5′-$OPO_3^{2-}$ | 3–8 | | 1.27 (6-H) 2.28 (5-$CH_3$) | 0.045 (N) 0.09 (N) 0.14 ($N^3$H) 0.04 (6-OH) | 2.0033 |
| 5′-dTMP[d] |  **15a** dr-5′-$OPO_3^{2-}$ | 7–10 | 1.62 (5-$CH_2$) 1.54 (5-$CH_2$) 1.05 (6-H) | | 0.12 (H) 0.04 (1 N) | 2.0023 |
| |  **15b** dr-5′-$OPO_3^{2-}$ | 10–11 | 1.64 (5-$CH_2$) 1.56 (5-$CH_2$) 0.098 (6-H) | | 0.04 (1 N) 0.04 (1′-H) | 2.0023 |
| 3′-dTMP[e] |  **16** dr-3′-$OPO_3^{2-}$ | 3–7 | – | 1.13 (6-H) 2.25 (5-$CH_3$) | – | 2.0032 |

[a] ± 0.005 mT; [b] ± 0.0001; [c] R = uracil–CH(OH)–CH(OH)–; [d] dr-5′-$OPO_3^{2-}$ = 2′-deoxyribose-5′-phosphate-1-yl; [e] dr-3′-$OPO_3^{2-}$ = 2′-deoxyribose-3′-phosphate-1-yl.

away from the plane of the sugar ring or over it [13].




The *anti* conformation is preferred by the majority of pyrimidine nucleosides and nucleotides, including those under investigation. In this conformation, the 5′-phosphate group closely approaches the alkenic bond of the pyrimidine ring, giving rise to characteristic NMR downfield shifts of the H(5)- or $CH_3$(5)- and H(6)-resonances of the of uracil [14] or thymine residues [15]. This situation strongly favours the addition of the phosphate group to the C(5)–C(6) double bond in radical reactions and thus the spectrum is assigned to the 5′,5-cyclic phosphate structure **10** (see Table III).

## 5′-UMP

As described earlier, reaction of $SO_4^{\cdot-}$ with the ribose-derivatives uridine [6], uridine-3′-phosphate (3′-UMP) [7] and polyuridylic acid [poly(U)] [7] results in the ESR spectra of sugar radicals. In the experiments with 5′-UMP, in addition to sugar radicals the 5′,5-cyclic phosphate adduct **11** was detectec (pH 2–11). The spectral parameters of **11** were identical with those of the corresponding radical **10** derived from 5′-dUMP. It is important to note that 5′-UMP was the only uracilyl ribose derivative to yield a base radical upon reaction with $SO_4^{\cdot-}$. This behaviour provides additional evidence for the specific role of the 5′-phosphate group in the radical reactions and confirms the assignment of radicals **10** and **11.**

Like in the case of uridine, in the experiments with 5′-UMP two different types of sugar radicals were detected. One of them (radical **12,** pH 2–8) was identified by the characteristic small doublet splittings of 1.36, 0.54 and 0.25 mT and the *g* factor of 2.0049. These parameters are identical with those of the 1′-radical **7** obtained in the experiments with uridine [6, 7]. The spectrum of the other sugar radical is similar to the open chain carbonyl conjugated species **8** derived from uridine at pH > 7 [6, 7]. It also exists in two conformations (**13a, 13b**) and is characterized by a doublet of triplets due to one α- and two equivalent β-protons, a small doublet due to one γ-proton and a small doublet splitting due to $^{31}P$. It was suggested that the partial double-bond character of the $^{\cdot}$–CH–CRO fragment gives rise to *E*- and *Z*-isomers [6, 7]. In agreement with the data on the radical $HO_3POCH_2$–$^{\cdot}$CH–CHO derived from glycerol-1-phosphate [16] the spectrum with the lower *g* factor was assigned to the *E* structure **13a**.

## 5′-dTMP

At pH 3–8 from solutions of 1 mM 5′-dTMP, 3 mM $K_2S_2O_8$ and 1% acetone, a spectrum of 8 groups of signals [a(H) = 1.27 mT, a($CH_3$) = 2.28 mT] of very weak intensity was observed (Fig. 2a). The signal: noise ratio could be improved by increasing the concentrations in the photolytical solution. By analysis of the two signal groups at

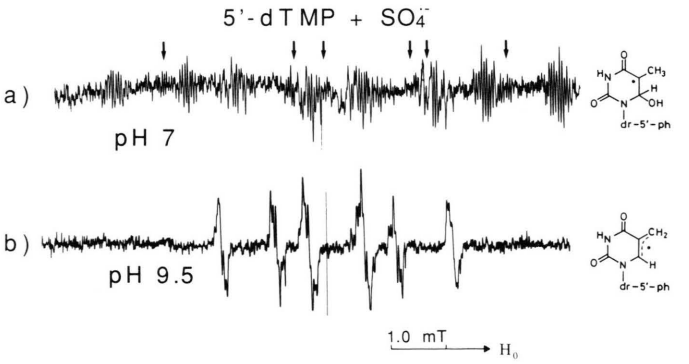



Fig. 2. ESR spectra obtained upon *in situ* photolysis of a solution containing a) 5′-dTMP ($10^{-3}$ mol × $l^{-1}$), peroxodisulfate ($5 \times 10^{-3}$ mol × $l^{-1}$) and acetone (1%). 277 K; a) pH 7; b) pH 9.5; arrows indicate contributions of additional unidentified radical(s).

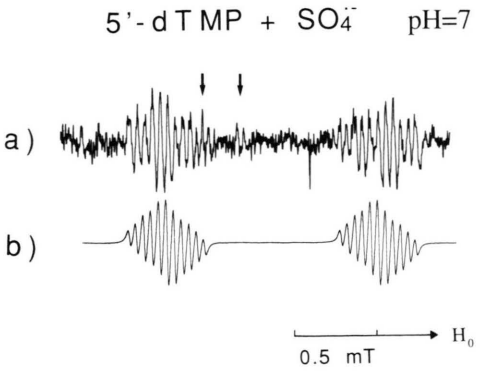



Fig. 3. Expanded region of the high field wing of the spectrum obtained from 5′-dTMP, $8 \times 10^{-3}$ mol × $l^{-1}$) with $K_2S_2O_8$ ($2 \times 10^{-2}$ mol × $l^{-1}$) and acetone (3%); a) experimental; b) simulated with the parameters given in Table III for radical **14;** arrows indicate contributions of additional, unidentified radical(s).

high field (Fig. 3), two nitrogen couplings of 0.09 and 0.045 mT and two doublet splittings of 0.14 mT [a(3-H)] and 0.04 mT [a(OH)] were uncovered. The values for a(5-$CH_3$) and a(6-H) and the *g* factor are in good agreement with the corresponding parameters of the OH adduct radical **6** derived from dT. Therefore, the spectrum was assigned to the 6-OH adduct radical **14.** In principle, the 5′,5-cyclic phosphate radical as described for 5′-dUMP and 5′-UMP should also be taken into account to explain the spectra in Fig. 2a and 3a. However, from the data in Table I it is obvious that the β-couplings are significantly lower for the phosphate adducts **3** and **4** as compared to those of the OH adducts **1** and **2** of 1-MeU and 1-MeT. Therefore, from the value of 1.27 mT for a(6-H) of radical **14,** intramolecular phosphate addition to the thymine ring is ruled out as an explanation for the spectrum.

Upon increasing the pH, the signals of **14** decreased in intensity and disappeared at pH 8. At pH > 7 the spectrum shown in Fig. 2b is detected. Its intensity increases with increasing pH values, reaching a maximum at pH ~ 10 and decreasing towards higher pH values, possibly because of the competing reaction of $SO_4^{\cdot-}$ with $OH^-$. The spectrum is assigned to the allyl-type radical **15** on the basis of the three characteristic doublet splittings and the *g* factor of 2.0023. Similar ESR parameters have been determined for the 5-methyleneuracil structures derived from thymine in aqueous solution at pH 13.3 [17] as well as from single crystals of thymine [18] and 1-MeT [19]. Like in the case of the OH-adduct radicals **5** and **9a–e** and of the cyclic phosphate radicals **10** and **11** deprotonation at N(3) resulted in the characteristic decrease of the couplings of **15** at pH ~ 10.

3′-dTMP

*In situ* photolysis of a solution containing $K_2S_2O_8$ and 3′-dTMP resulted in a doublet of quartets with coupling constants of 1.13 mT [a(6-H)] and 2.25 mT [a($CH_3$)] with $g = 2.0032$. These parameters are almost identical with those of radicals **6** and **14** obtained under similar conditions from dT [6] and 5′-dTMP. Therefore, the spectrum is assigned to the 6-OH-5-yl radical **16.** The signal intensity of **16** reached a maximum at pH 4. At about pH 7 the spectrum had disappeared and, in contrast to 5′-dTMP, there was no evidence for an allyl-type radical at pH > 7.

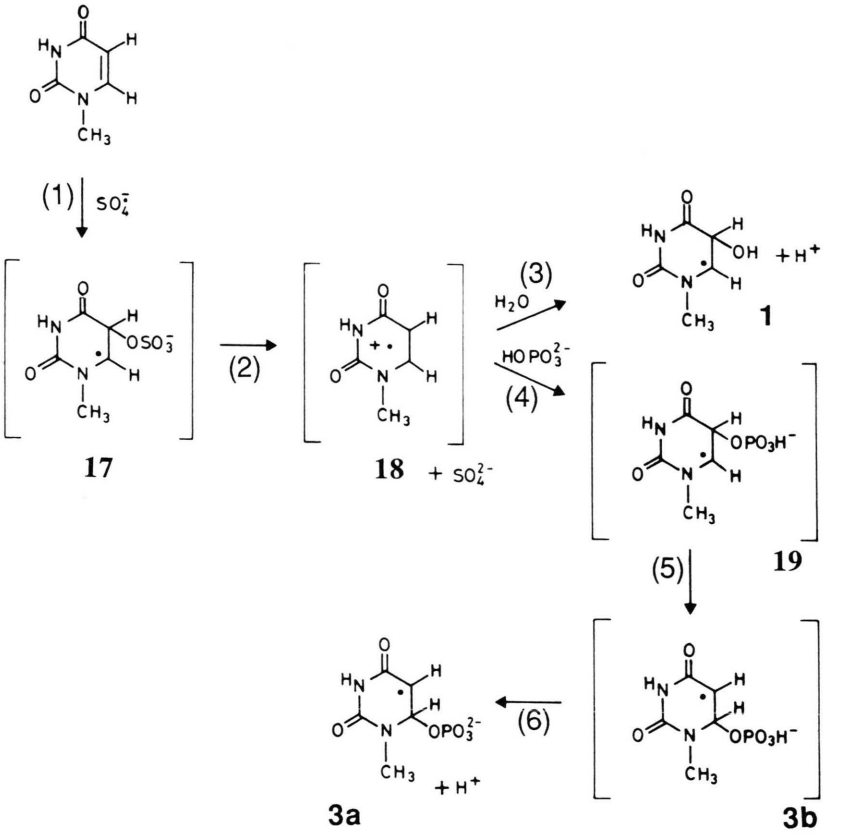
*Pulse conductivity*

Pulse radiolysis measurements were performed at pH 4.5 with a solution containing dU (1 mM), peroxodisulfate (10 mM) and *tert.*-butanol (50 mM) as a scavenger for OH radicals. The conductivity increase after the pulse corresponded to the release of one proton and it occurred in a time interval of less than 1 μs. Pulse conductivity experiments with dT showed similar results [20]. From measurements with the nucleotides relevant data are not expected because of the buffering capacity of the phosphate group.

## Discussion

There are characteristic differences in reactions induced upon interaction of $SO_4^{\cdot-}$ with 1-MeU, 1-MeT, dU, dT and 3′-dTMP on the one hand, and with 5′-dUMP, 5′-UMP and 5′-dTMP on the other hand. From MeU and dU the 5-OH adduct radicals **1** and **5** were generated whereas in the experiments with 5′-dUMP and 5′-UMP the intramolecular 5′,5-cyclic phosphate radicals **10** and **11** were detected. All the thymine derivatives, including 5′-dTMP gave rise to 6-OH adduct radicals at pH < 7. From 1-MeT, dT and 3′-dTMP at pH > 7 no ESR signals were detected whereas from 5′-dTMP the signals of the 5-methyleneuridine-5′-phosphate radical **15** were obtained. As shown for 1-MeU in Scheme 3 it is assumed [5] that in the

primary step [reaction (1)] the sulfate adduct radical **17** is produced from pyrimidine derivatives which by elimination of the sulfate group [reaction (2)] may yield the radical cation **18.** The OH adduct radical **1** is generated by hydrolysis [reaction (3)]. In the presence of phosphate dianions the 5-phosphate adduct radical **19** is formed in reaction (4). It rearranges by a rapid 1,2-phosphate shift [reaction (5)] to the 6-phosphate adduct **3** which stabilizes by deprotonation [reaction (6)]. Formation of the OH adduct radical **5** from dU and of the phosphate adduct **3** from 1-MeU is in line with the reactions (1)–(6).

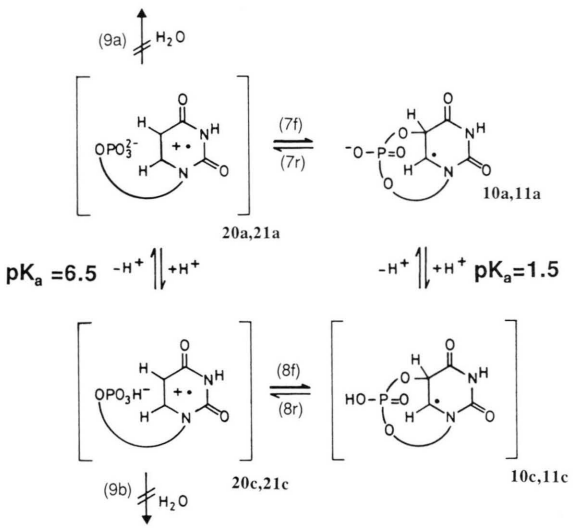



In the thymine series due to the presence of the 5-methyl group, reaction of $SO_4^{\cdot-}$ leads to the 6-OH adduct radicals (radicals **2, 6, 14** and **16**). The phosphate dianion adds at C(6) of the thymine radical cation and the phosphate adduct **4** from 1-MeT is probably generated directly and not necessarily *via* addition to C(5) and subsequent 1,2-phosphate shift. The failure to detect thymidyl OH adducts at pH > 7 does not necessarily imply that these radicals are not generated in alkaline solution but may rather be due to their fast decay in secondary reactions.

It was shown by pulse radiolysis that the OH adduct radical of 1,3-dimethyluracil was formed in less than 1 μs, *i.e.* the lifetimes of the sulfate adduct radical and of the radical cation are therefore shorter than this time-span [21]. Similar results have been obtained for dU (this work) and also for dT [20]. This means that, in general, in fluid aqueous solution the sulfate adducts and the radical cations of pyrimidine derivatives are too short-lived to be characterized by ESR spectroscopy. Only in the case of tetramethyluracil was it possible to detect by laser flash photolysis a short-lived optical transient ($\tau_{1/2} \sim 10$ μs), which was assigned to the radical cation [7, 22]. At low temperatures, ESR spectroscopic evidence for pyrimidine radical cations was obtained by X-irradiation in sulfuric acid glasses [23] and upon γ-irradiation in $CFCl_3$ at 77 K [24].

*Intramolecular phosphate addition in 5′-dUMP and 5′-UMP*

Intramolecular phosphate addition in the 5′-nucleotides is strongly favoured by the close approach of the phosphate group and the base moiety in the *anti* conformation (see Scheme 2). Obviously, heterolytic decay of the 5′,5-cyclic phosphate radicals **10** and **11** [reactions (7r) and (8r)] is much slower than the forward reactions (7f) and (8f) and the OH adduct radical formed in hypothetical reactions (9a) and (9b) is not detected.




The 1,2-phosphate shift analogous to reaction (5) is not observed for the 5′,5-cyclic phosphate radicals **10** and **11** possibly because of steric constraints within the cyclic species.

Whereas the ESR signals of **10** and **11** were detected at pH 2–11 those of the phosphate adducts of 1-MeU and 1-MeT were observed at pH >6.5, only. This difference requires some comment.

From studies of Behrens *et al.* [25] it is well-known that the stability of β-phosphate adduct radicals depends on the state of protonation and alkylation of the phosphate group. The 2-yl radical **22** derived from 2-methoxyethylphosphoric acid has two $pK_a$ values:

$CH_3-O-\dot{C}H-CH_2-OPO_3H_2$ **22a** $pK_a \sim 1.5$

$CH_3-O-\dot{C}H-CH_2-OPO_3H^- + H^+$ **22b** $pK_a \sim 6.5$

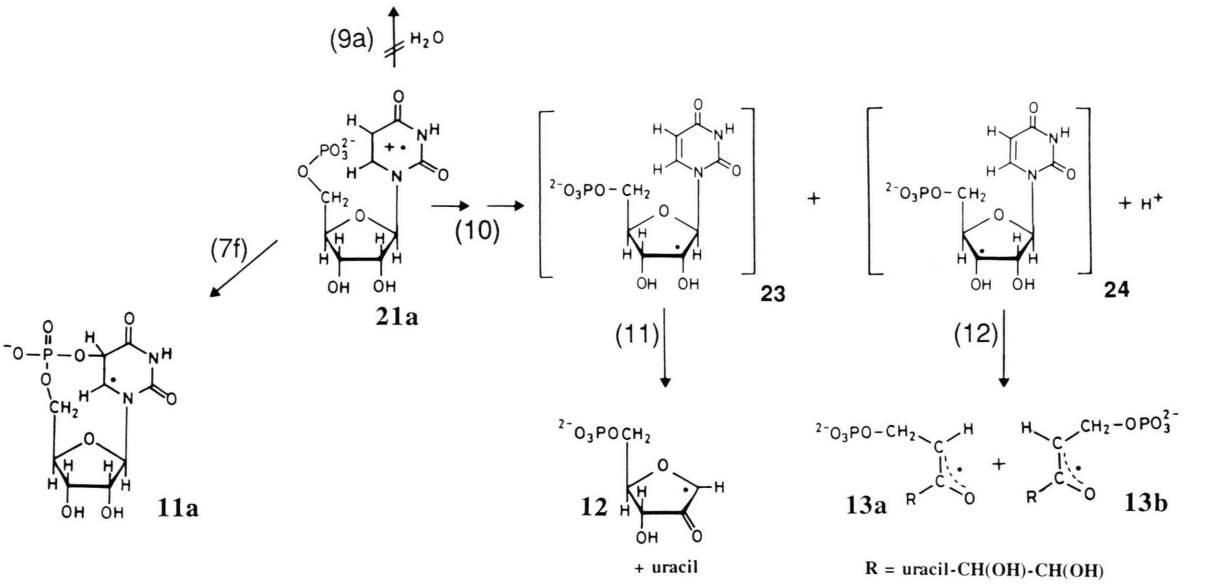
$CH_3-O-\dot{C}H-CH_2-OPO_3^{2-} + H^+$ **22c**

The rates of heterolytic decay were $3 \times 10^6$ $s^{-1}$ for **22a**, $10^3$ $s^{-1}$ for **22b** and 0.1–1 $s^{-1}$ for **22c.** From kinetic considerations [26] it is known that radicals with lifetimes $<10^{-3}$ s are not detectable by steady-state ESR. This explains why radicals **22b** and **22c** were observed whereas no signals were obtained from **22a.** Accordingly, only the dianion form of the phosphate adduct radicals **3** and **4** of 1-MeU and 1-MeT gave rise to ESR spectra at pH >6.5 [5]. The monoanions and the neutral forms were too short-lived for ESR detection.

As far as the 5′,5-cyclic phosphate radicals **10** and **11** are concerned stabilization by release of a proton is achieved at pH ~1.5 (see Scheme 4). The monoanions **10a** and **11a** were detected and no change in ESR intensity at pH ~6.5 is expected like in the case of the phosphate adducts **3** and **4**.

*Sugar radicals **12** and **13** derived from 5′-UMP*

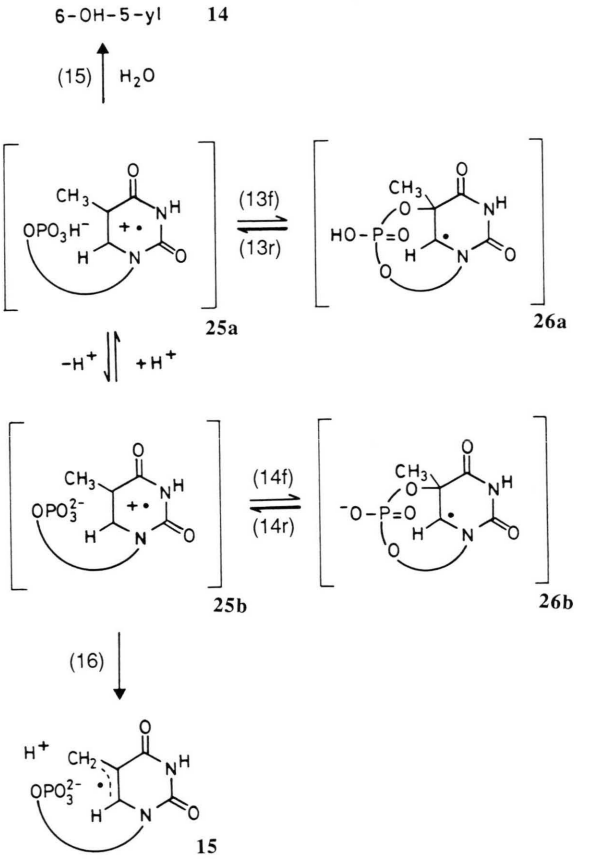
As described earlier [6, 7], reaction of $SO_4^{\cdot-}$ with deoxyribose derivatives of uracil or cytosine resulted in the ESR spectra of base radicals whereas for the ribose derivatives sugar radicals were obtained. 5′-UMP was the only substrate which gave rise to an overlap of ESR signals of a base radical (radical **11**) and sugar radicals (radicals **12** and **13**). The rate constant for reaction of $SO_4^{\cdot-}$ with the pyrimidine moiety [27] ($k \sim 10^9$ l $mol^{-1}$ $s^{-1}$) is significantly larger than for H abstraction from alcohols and ethers ($k_{abs} \sim 10^6-10^8$ l $mol^{-1}$ $s^{-1}$) [28]. Therefore, it is concluded that in nucleosides and nucleotides the primary step is reaction of $SO_4^{\cdot-}$ with the base moiety [6, 7]. For the deoxyribose derivatives the reactions presented in Scheme 3 for 1-MeU are observed whereas in the ribose derivatives the site of the free spin is transferred from the base to positions C(2′) and C(3′) of the sugar moiety [reaction (10)] [6, 7]. Like in the case of uridine, the 2′-radical **23** of 5′-UMP leads to elimination of uracil with formation of radical **12** [reaction (11)] and the 3′-radical **24** undergoes ring opening [reaction (12)] at pH >7 thus yielding the open chain species **13a** and **13b.** In 5′-UMP intramolecular addition (7f) of the 5′-phosphate group to the base cation is fast enough to compete with the intramolecular formation (10) of sugar radicals.




*Radicals derived from 5′-dTMP: 5-OH adduct and allyl radical*

In contrast to 5′-dUMP and 5′-UMP, the thymine nucleotide 5′-dTMP did not give rise to ESR signals of the 5′,5-cyclic phosphate radical. Instead, at pH 2–8, the 6-OH adduct radical **14** and at pH 7–11 the allyl-type radical **15** were detected. It may be assumed that by the presence of the methyl group the radical cation **25** is stabilized and the rate of heterolytic decay [reactions (13r) and (14r)] of the 5′,5-cyclic phosphate radical **26** is increased. In fact, upon substitution of an α- or β-methyl group instead of a H atom, radicals with electronegative β-substituents show an increase in the rate of heterolytic cleavage by several orders of magnitude [29]. The radical cation **25a** reacts predominantly with $H_2O$ to yield the OH adduct **14** [reaction (15)] whereas by the strongly basic phosphate dianion group in **25b** deprotonation of the base radical cation is favoured [reaction (16)].

To my knowledge this is the first example of phosphate-catalyzed deprotonation of a carbon atom of a radical cation while the catalytic effect of phosphate in the reserve type of reaction, *i.e.* in protonation of carbon atoms of radical anions, has been well documented [30].



The assumption of formation of a short-lived 5′,5-cyclic phosphate radical **26** provides the possibility of several repetitive steps of phosphate addition and elimination [reactions (13) and (14)] and, eventually, loss of the proton in reaction (16). In analogy to this pathway, generation of an allyl-type radical from 2,3-dihydrofuran was explained by Gilbert *et al.* [31] and Behrens *et al.* [32]. It was suggested that, at low pH, during a cycle of water readdition and reelimination steps a proton should be released from the radical cation of the vinyl ether.

It has been mentioned above (see Results) that there is already ESR spectroscopic evidence for formation of the thymidyl allyl-type radical not only in the solid state [18, 19] but also in aqueous solution. *In situ* radiolysis of thymine at pH 13.3 [17] resulted in the corresponding radical anion and more recently upon γ-irradiation of dT and of DNA in neutral solutions, Kuwabara *et al.* [33] detected the 5-methyleneuracil species by spin-trapping. Also at neutral pH by pulse radiolysis evidence for the allyl-type radical of thymine was obtained [10]. It is generally accepted that in those experiments hydrogen abstraction from the thymidyl methyl group by $O^{\cdot-}$ or by OH radicals was the crucial step. From the characteristic pH dependence of the ESR spectra in Fig. 2 and from the failure to detect an allyl-type radical upon reaction of $SO_4^{\cdot-}$ with 3′-dTMP, direct hydrogen abstraction from 5′-dTMP by $SO_4^{\cdot-}$ is ruled out as an explanation for the results of the present paper. Instead, one may conclude that the spectrum in Fig. 2b is evidence for deprotonation of the 5-methyl group of the thymidyl radical cation.

A similar reaction pathway is postulated in a series of studies using product analysis to show the intermediate formation of an allyl-type radical under conditions which seem to favour the generation of a thymidyl radical cation from dT or 1,3-dimethylthymine. This was achieved either with the help of photoexcited 2-methyl-1,4-naphthoquinone [34] or with $SO_4^{\cdot-}$ [35] or by γ-irradiation in frozen aqueous solution [36]. In contrast to the ESR studies the presence of the 5′-phosphate group was not essential for formation of the allyl-type radical. This might indicate that in 5′-dTMP by the phosphate group a delicate balance of reactions is shifted in a way which raises the steady state concentration of the allyl-type radical above the level required for ESR detection.

## Materials and Methods

5′-dUMP, 5′-dTMP and 3′-dTMP were obtained from Sigma and used without further purification. $K_2S_2O_8$, p.a., was from Merck, Darmstadt.

*ESR experiments*

a) Photolytical generation of radicals: Experiments were performed at 277 K on aqueous solutions containing the substrates (1–3 mM) and $K_2S_2O_8$ (3 mM). By a continuous flow arrangement the solutions were pumped through the ESR quartz cell. The standard flow rate was 0.1 ml $s^{-1}$ for a 0.3 × 8 $mm^2$ cross-section. *In situ* photolysis was achieved by irradiation with a high-pressure mercury lamp (Philips SP 1000). The solutions were gassed with argon 0.5 h prior to the measurement and during the measurement. The pH values were adjusted with KOH or $HClO_4$. At pH > 7 sodium borate was used as a buffer. 1% acetone was added to the solutions to increase the spectral intensity. The acetone did not give rise to any signals which were not detected in the absence of the photosensitizer. Spectra were measured at the X-band with 100 kHz modulation. The *g* factors were determined with a NMR sideband technique [37] taking into account the field offset of the NMR probe that was attached to the side-wall of the cavity.

b) Radiolytic generation of ·OH radicals: In the photolysis experiments the quality of the spectra of the OH adduct radicals **5** and **9a–e** decreased at pH > 7. In this pH region the radiolysis method yields much better results. For this purpose the radicals were generated in the cavity of a Varian E-9 ESR spectrometer by *in situ* irradiation with a beam (diameter 1 mm) of 2.4 MeV electrons in a set-up similar to that described by Eiben and Fessenden [38]. The concentration of the substrates was 1–3 mM. pH values were adjusted with NaOH or $HClO_4$. The solutions were saturated with $N_2O$ by gassing for 40 min, prior to and during the measurements. $N_2O$ was freed from oxygen by passage through columns packed with chromosorb, Messer-Griesheim, Duisburg. The centers of the spectra were overlapped by the intense signal

of the quartz cell. Therefore, only the sum a(H-α + a(H-β) could be evaluated whereas the individual values for the α- and β-proton couplings were not obtained.

*Pulse conductivity*

Electron pulses of 1 μs duration were provided by a 3 MeV van de Graaf accelerator. The conductivity change after the pulse was measured with an AC powered bridge (time resolution ~ 3μs) and a DC bridge (time resolution ~ 1μs) [39]. The solutions containing 1 mM dU. 10 mM $K_2S_2O_8$ and 50 mM *tert.*-butanol were gassed with Ar (pH 4.5). Dosimetry was performed with $(CH_3)_2SO$ (10 mM) in $H_2O$, saturated with $N_2O$ (pH 4.5).

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