Pattern of OH Radical Addition to Cytosine and 1-, 3-, 5-, and 6-Substituted Cytosines. Electron Transfer and Dehydration Reactions of the OH Adducts

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Abstract: By use of the technique of pulse radiolysis with optical detection, the isomer distribution of the radicals formed in aqueous solution by addition of OH radicals to cytosine, 5-methylcytosine, 5-carboxylcytosine, 3-methylcytosine, 1-methylcytosine, cytidine, 5-methylcytidine, cytidylic acid, 2'-deoxycytidine, 2'-deoxycytidylic acid, and 2-amino-4-hydroxy-6-methylpyrimidine has been determined by utilizing differences between the isomeric OH adducts with respect to electron-transfer reactions with the reductant *N,N,N'N'*-tetramethyl-*p*-phenylenediamine (TMPD) or the oxidant tetranitromethane (TNM). The radicals Cy-5-OH, formed by addition of OH to C(5) of cytosine (87% of the OH radicals), 3- and 5-methylcytosine (92% and 65%, respectively), 5-carboxylcytosine (82%), and 2-amino-4-hydroxy-6-methylpyrimidine (95%) reduce TNM to yield nitroform anion. The radicals Cy-6-OH, formed by addition to C(6), oxidize TMPD to yield $TMPD^{+}\cdot$. The Cy-5-OH radicals undergo a base-catalyzed dehydration reaction to yield radicals that are able to oxidize TMPD to yield $TMPD^{+}\cdot$. In the case of cytosine it is shown that the dehydrated OH adduct is identical with the one-electron oxidation product from the reaction of cytosine with $SO_4^{-}\cdot$ and that the pK_a of this radical is 9.6. If N(1) of the pyrimidine ring is substituted as with 1-methylcytosine, cytidine, cytidylic acid, 2'-deoxycytidine, and 2'-deoxycytidylic acid, no dehydration reactions of the OH adducts occur. In contrast, substitution by alkyl at N(3) does *not* inhibit the dehydration reaction of the corresponding Cy-5-OH radical. In the presence of oxygen the Cy-5-OH and Cy-6-OH radicals are converted into peroxyl radicals which oxidize TMPD with rate constants of $1.6 \times 10^8\ M^{-1}\ s^{-1}$. In basic solution these peroxyl radicals decompose, presumably by elimination of $O_2^{-}\cdot$. With 5-methylcytosine a peroxyl radical derived from the radical formed by H abstraction from the methyl group is additionally observed. It contributes ≈10% to the production of $TMPD^{+}\cdot$. With the cytosine nucleosides and nucleotides the probability of OH attack at the cytosine molecule is >80%.

Introduction

The OH radical, the most reactive of the three radical species produced in the radiolysis of aqueous systems, has been shown[2] to react with pyrimidines such as uracil, thymine, and cytosine by addition. With uracil and thymine there is only one site for addition, i.e., the C(5)–C(6) double bond. With cytosine, however, the double bond beween N(3) and C(4) is a potential additional reaction site. Electron spin resonance,[3-7] pulse radiolysis with optical[8-12] and conductometric[12] detection, and product analysis[2] techniques have been applied in order to determine the pattern of OH addition to cytosine. The results support the view that, as with uracil and thymine,[2-7,13,14] OH addition takes place predominantly at C(5)/C(6),[6,9,12] with a preference for C(5).[6,12] However, it has so far not been possible to determine the percentages of the isomeric radicals produced and thus establish a material balance that accounts quantitatively for the fate of the OH radical. The present study was undertaken to achieve this by using specific redox scavengers for the isomeric radicals.

The radicals produced by OH reaction with cytosine undergo OH^{-}-induced changes[8,9,11,12] that follow first-order kinetics.[8,12] These changes have been interpreted as due to ionization,[8] to ring opening,[9] or to rearrangement.[12] The ionization hypothesis[8] can be excluded by kinetic arguments,[12] and the ring-opening mechanism[9] is possible only for a radical that, as will be shown later, is formed in minor proportion. All three hypotheses[8,9,12] are unable to explain the change with pH in the redox properties observed[11] polarographically.

It has been shown recently[14,15] that in the case of OH adduct radicals of uracils, a dehydration reaction takes place that converts a reducing radical into an oxidizing one.[14] A similar reaction has also been observed with OH adducts of pyridones,[16] which may be considered model compounds for oxopyrimidines. The present study was performed in order to see whether this type of reaction occurs also with the cytosines and and to investigate the effects of substitution at the cytosine system on that reaction.

Experimental Section

The substrates were obtained from Aldrich, Fluka, Sigma, and Vega Biochemicals and were used as received. The solutions typically contained 2 mM cytosine or its derivatives in triply distilled water, and they were saturated with N_2O in order to convert e_{aq}^{-} into OH. When the production of oxygenated radicals was required, the solutions were saturated with a 4:1 mixture of N_2O and O_2. The pH of the solutions was adjusted with NaOH or phosphate buffer. With use of a 3-MeV van de Graff accelerator the solutions were irradiated at room temperature (20 ± 2 °C) with electron pulses of 0.4–1-μs duration with doses such that 1–2 μM radicals were produced. The photomultiplier signal was digitized by using a Biomation type 8100 transient recorder. A data collection program was run on a VAX 780 computer with the transient recorder connected to a PDP 11/10 minicomputer, which served as an intelligent terminal. The minicomputer controlled the transient recorder and performed data reduction to shorten the time needed for transmission of the data to the VAX 780. The experimental data were stored, processed, and analyzed with the VAX 780.

Dosimetry was performed with N_2O-saturated 10 mM KSCN solutions taking $\epsilon(SCN)_2^{-}\cdot_{480\ nm}$ 7600 $M^{-1}\ cm^{-1}$ and $G(OH) = 6.0$. In solutions containing a substrate S that can compete with N_2O for e_{aq}^{-}, $G(OH)$ is reduced to an extent that depends on [S] and $k(e_{aq}^{-} + S)$. For example, for N_2O saturated solutions of cytosine, $G(OH) = 5.9$ if [cytosine] = 0.5 mM and it is 5.7 if [cytosine] = 2 mM. The yields per OH of radicals reported in Tables II and III and in Figure 2 were calculated by using these numbers.

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Table I. Reaction of OH with Cytosine. Dependence on pH of the Total Yield[a] of $TMPD^{+\cdot}$ and of k_{deh}, the Rate Constant for Dehydration of Cy-5-OH (=OH^--Induced Formation of $TMPD^{+\cdot}$)[b]

pH	$G(TMDP^{+\cdot})$	k_{deh}/s^{-1}
7.5	1.2 ± 0.2	$\leq 10^3$
9	2.9	7.7×10^3
10	4.2	3.6×10^4
11	4.6	1.8×10^5
12	4.8	3.5×10^5
13	4.9	4.4×10^5

[a] Expressed as G value. The G value measures the number of molecules (trans)formed per 100 eV of absorbed radiation. [b] [Cytosine] = 2 mM, [TMPD] = 0.05–0.5 mM; N_2O saturated.

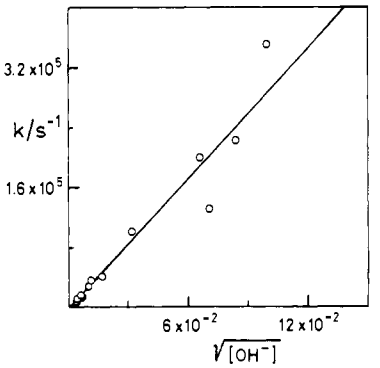

Figure 1. Dependence on pH of k_{deh}, the rate constant for the OH^--induced dehydration of Cy-5-OH. The slope corresponds to 2.9×10^6 s^{-1} $[OH^-]^{-1/2}$.

The yields of oxidizing and reducing radicals were determined by using *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD) and tetranitromethane (TNM), respectively, and taking[14] $\epsilon(TMPD^{+\cdot})_{565\,nm}$ 12 500 M^{-1} cm^{-1} and $\epsilon(C(NO_2)_3^-)_{350\,nm}$ = 13 250 M^{-1} cm^{-1}.

1. Results

1.1. Oxidizing Radicals Produced by Reaction with OH in N_2O-Saturated Solutions. OH radicals, produced by pulse irradiation of the aqueous solutions, were reacted with cytosine (2 mM) in the presence of 0.05–0.2 mM *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD), and the optical density was monitored at 565 nm, where $TMPD^{+\cdot}$ has an absorption maximum. At pH 7–8 the reaction results in the production of $TMPD^{+\cdot}$. There is a small step, due to the direct production of $TMPD^{+\cdot}$ by OH[17,18] (see section 2.1), followed by a slower and exponential further increase of $[TMPD^{+\cdot}]$, which reflects the reaction of cytosine-derived radical(s) with TMPD. From the dependence of the rate of formation of $TMPD^{+\cdot}$ on [TMPD] in this slower process, the rate constant for reaction of TMPD with the cytosine-derived radical Cy-6-OH (see section 2.1), k(Cy-6-OH + TMPD), is obtained as 1.1×10^9 M^{-1} s^{-1}.

At pH 8.5–9.5, in addition to the directly formed $TMPD^{+\cdot}$, *two* components become visible in the buildup kinetics of $TMPD^{+\cdot}$. After the initial slow growth of optical density, which is the same as that at pH 7–8, there is a further increase, the rate of which is *independent* of [TMPD] (in the range of 0.05–0.2 mM) but dependent on pH. As shown in section 2.3, this OH^--induced reaction involves the dehydration of Cy-5-OH. Table I shows that the rate constant for this reaction, k_{deh}, increases from 7.7×10^3 at pH 9 to 4.4×10^5 s^{-1} at pH 13. As seen from Figure 1, the dependence on pH of k_{deh} may be expressed by the empirical relation $k_{deh} = 2.9 \times 10^6[OH^-]^{1/2}$ s^{-1}. At pH >9 the pH-dependent component in the production of $TMPD^{+\cdot}$ becomes so dominant that the pH-*independent* process is completely masked. At pH 13 the rate constant for oxidation of TMPD by the cytosine-derived radical $Cy^{\cdot}$ (see section 2.3) is 2×10^9 M^{-1} s^{-1}.

As a result of the OH^--induced reaction, the yield of $TMPD^{+\cdot}$ increases with pH. Figure 2 shows the pH dependence of the ratio

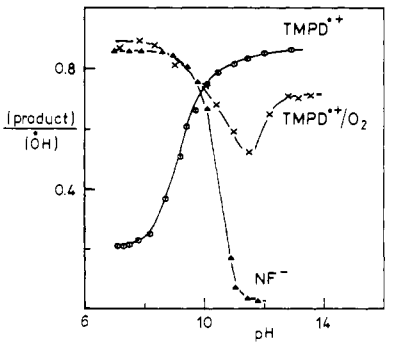

Figure 2. Dependence on pH of the yields per OH of oxidizing (measured as $TMPD^{+\cdot}$) and reducing radicals (measured as NF^-). o (2 mM cytosine, 0.2 mM TMPD) and ▲ (0.5 mM cytosine, 1 mM TNM): solutions saturated with N_2O. ×: (2 mM cytosine, 0.2 mM TMPD), solution saturated with 4:1 N_2O/O_2. The yields of $TMPD^{+\cdot}$ include those from the direct reaction OH + TMPD (see text).

$TMPD^{+\cdot}$/OH for a dose rate of ≈200 rd/pulse. The yield of $TMPD^{+\cdot}$ increases with increasing pH to a maximum value at pH ≈13, which corresponds to 87% conversion of the OH radicals to yield $TMPD^{+\cdot}$. In order to correct this number for loss due to radical–radical reactions (which compete with the formation of $TMPD^{+\cdot}$), a dose rate variation was performed at pH 12.2, and from the data the yield per OH of $TMPD^{+\cdot}$ calculated[19] for zero initial radical concentrations is 1.02 ± 0.05. This shows that at pH 12.2 the radicals formed by reaction of OH with cytosine convert TMPD into $TMPD^{+\cdot}$ quantitatively.

Results analogous to those described for cytosine were obtained on reaction of OH with 3-, 5-, and 6-methyl- and with 5-carboxyl-substituted cytosines in the presence of TMPD. For these compounds and also for N(1)-substituted cytosines (the cytosine nucleosides and nucleotides), Table II shows the yields and the rate constants (k(Cy-6-OH + TMPD)) for formation of $TMPD^{+\cdot}$ via the initial components, as measured at pH 7–8. Except for substitution by carboxyl at C(5) and by methyl at N(3), the *rate constants* are decreased by substitution of the cytosine ring. The fact that k(Cy-6-OH + TMPD) is higher for 5-carboxylcytosine than for cytosine and 5-methylcytosine shows that the increase is due to electronic factors. (CO_2^- is slightly electron withdrawing, thereby increasing the electron deficiency of Cy-6-OH. The opposite is true for methyl.) The decrease observed on substitution of H at N(1) by methyl, deoxyribose, or deoxyribose phosphate moieties probably results from a combination of electronic and steric factors.

As compared to cytosine, the *yields* of $TMPD^{+\cdot}$ produced via the initial component are increased by ≈50% if C(5) is substituted by a methyl or carboxyl group. In contrast, substitution by methyl at C(6) *decreases* the $TMPD^{+\cdot}$ yield by ≈50%, while substitution at N(1) or N(3) has little influence on the yields of $TMPD^{+\cdot}$.

With the cytosines methylated at N(3), C(5), or C(6), the OH^--induced reaction that leads to the additional production of $TMPD^{+\cdot}$ becomes visible starting already at pH 7.5–8. At the same pH, the rates of this reaction (see Table II) are considerably faster than with unsubstituted cytosine or with analogous substituted uracils.[14] For instance, for 6-methylcytosine the k_{deh} vs. $[OH^-]^{1/2}$ plot has a slope of 3.9×10^7, to be compared with 2.9×10^6 for cytosine. In contrast, substitution of H at C(5) by CO_2^- results in a pronounced decrease in the rate of dehydration, such that it becomes visible only above pH 11.

In contrast to the cytosines substituted at N(3), C(5), or C(6), the OH^--induced reaction is absent with cytosines substituted at N(1). With these compounds, up to pH 11 the yield of $TMPD^{+\cdot}$ is equal with that at pH ≈7. However, at pH 12 there is a 10–20% increase, and at pH 13 the yield of $TMPD^{+\cdot}$ is approximately twice that at pH 7 but still far from complete. The changes in the $TMPD^{+\cdot}$ yield at pH 12–13 are probably due to conversion of OH into $O^{-\cdot}$ radical, which occurs in this pH range (pK_a(OH) = 11.9).

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Table II. Yields and Rates of Reaction of Cy-6-OH with TMPD

pyrimidine	$G(TMPD^{+\cdot})_{obsd}$[a]	[Cy-6-OH]/[OH][b]	k(Cy-6-OH + TMPD)/M^{-1} s^{-1}	k_{deh}/s^{-1} and $G(TMPD^{+\cdot})$[c] at pH 10
cytosine	1.2 ± 0.2	0.10	1.1×10^9	3.6×10^4 (4.2)
3-methylcytosine	1.2 ± 0.2	0.10	1.1×10^9	6.0×10^4 (4.6)
5-methylcytosine	1.8	0.22	4.2×10^8	1×10^5 (3.4)
5-carboxylcytosine	≈1.9	≈0.24	1.6×10^9	*d* (1.9)
6-methylisocytosine[e]	0.9	0.04	3.6×10^8	3.9×10^5 (1.9)
1-methylcytosine	1.1	0.08	7.2×10^8	*d* (1.1)
cytidine	1.4	0.14	5.1×10^8	*d* (1.5)
2'-deoxycytidine	1.2	0.10	4.1×10^8	*d* (1.2)
cytidylic acid	1.1	0.08	4.3×10^8	*d* (1.1)
2'-deoxycytidylic acid	1.1	0.08	4.4×10^8	*d* (1.1)
5-methylcytidine	≈2	≈0.26	3.3×10^8	*d* (2)

[a] pH 7–8, [TMPD] = 0.2 mM, [pyrimidine] = 2 mM; dose rate ≈200 rd/pulse. The yields are accurate to ±10%. [b] Corrected for the $TMPD^{+\cdot}$ produced by the direct reaction OH + TMPD ($[G_{obsd} - 0.7]/5.0$. [c] *G* values in brackets. [d] A base-catalyzed reaction is not seen; if it occurs, its rate is $<10^3$ s^{-1}. [e] ≡2-Imino-4-hydroxy-6-methylpyrimidine.

Table III. Yields and Rates of Reaction with TNM of Cy-5-OH and Total Yields of Radicals[a]

pyrimidine	$G(NF^-)$[b]	[Cy-5-OH]/[OH][c]	k(Cy-5-OH + TNM)/M^{-1} s^{-1}	([Cy-5-OH] + [Cy-6-OH])/[OH][d]
cytosine	5.1	0.87	1.1×10^9	0.97
3-methylcytosine	5.4	0.92	9.0×10^8	1.02
5-methylcytisone	3.8	0.65	1.6×10^9	0.88
5-carboxylcytosine	4.8	0.82	1.1×10^9	1.07
6-methylisocytosine	5.6	0.95	1.4×10^9	0.99
1-methylcytosine	5.1	0.87	8.8×10^8	0.95
cytidine	5.2	0.89	6.0×10^8	1.03
2'-deoxycytidine	5.2	0.88	5.6×10^8	0.98
cytidylic acid	5.2	0.88	2.0×10^8	0.96
2'-deoxycytidylic acid	5.1	0.87	2.6×10^8	0.95
5-methylcytidine	3.9	0.66	4.0×10^8	0.93

[a] [Pyrimidine] = 0.5 mM, [TNM] = 0.5–1 mM, pH 7–8, dose rate ≈200 rd/pulse. [b] These values are accurate to ±10%. The values may contain a ≤10% contribution due to H atoms. In the worst case, this leads to a reduction of the Cy-5-OH/OH values by 10% (see text). [c] Taking $G(OH) = 5.9$. [d] The values for Cy-6-OH are from Table II.

1.2. Reducing Radicals Produced by Reaction with OH. N_2O-saturated aqueous solutions containing 0.5 mM cytosine or its derivatives and 0.2–1 mM tetranitromethane (TNM) were pulse irradiated. Under these conditions the OH radicals produced by the pulse react quantitatively with the cytosine. After initiation of the reaction, the formation of nitroform anion, $C(NO_2)_3^-$ (NF^-), was observed at 350 nm, where NF^- has an absorption maximum. The (first-order) rate of formation of NF^- depended linearly on the TNM concentration, and from this dependence the rate constants for the bimolecular reaction between the cytosine-derived radical(s) and TNM were obtained (Table III). For the substituted cytosines, the rate constants are between 2×10^8 and 2×10^9 M^{-1} s^{-1}. Table III contains also the yields of NF^-. The yields of NF^- are less than that of OH and depend on the structure of the cytosine: the yields are decreased by substitution with methyl at C(5) but increased if C(6) is methylated. This dependence on structure is opposite to that observed with TMPD (which measures the oxidizing radicals (see section 1.1)). Surprisingly, substitution at C(5) by CO_2^- does not reduce the NF^- yield appreciably. Substitution at N(3) and N(1) increases the yield of NF^- slightly; however, the rate constants for reaction with TNM decrease considerably, especially if N(1) carries the bulky deoxyribose or deoxyribosyl phosphate groups.

Figure 2 shows the pH dependence of the yield per OH of NF^- obtained on reaction of OH with cytosine in the presence of TNM. The yield of NF^- decreases drastically between pH 9 and 10.5, and at pH 12 it is ≤0.03. This pH dependence is opposite to that for the production of $TMPD^{+\cdot}$, which shows that the reducing radical formed by OH reaction with cytosine is converted into an oxidizing one by an OH^--induced reaction.

The OH^--induced conversion of a reducing radical into an oxidizing one is also observed with *N*(3)-methylcytosine, but not with any of the N(1)-substituted cytosines. With these the yield of NF^- is essentially unchanged between pH 6 and ≈11. At higher pH values measurements cannot be performed due to rapid decomposition of TNM.

1.3. Oxidizing Radicals Produced by OH in the Presence of O_2. The solutions contained 2 mM (substituted) cytosine and 0.05–0.2 mM TMPD, and they were saturated with a 4:1 (v/v) mixture of N_2O and O_2. Under these conditions the concentration of O_2 is 0.28 mM, a value too low for O_2 to compete with N_2O for e_{aq}^- but sufficiently high[20] to ensure complete reaction between O_2 and organic radicals. When OH was reacted with, e.g., cytosine at pH 7, the production of $TMPD^{+\cdot}$ was observed. Different from the uracil system previously studied,[14] there was only a single component in the (exponential) buildup of $TMPD^{+\cdot}$. The rate of formation of $TMPD^{+\cdot}$ was linearly dependent on [TMPD], i.e., the rate-determining step involves reaction of cytosine-derived radicals with TMPD. At pH 7–8, the rate constant measured for formation of $TMPD^{+\cdot}$ is 1.6×10^8 M^{-1} s^{-1}, i.e., 7 times less than that in the absence of O_2; however, the yield of $TMPD^{+\cdot}$ was 90%. The same yield was obtained with 5-methylcytosine. At the dose rates used (≈200 rd/pulse), the 90% yield corresponds to quantitative conversion of OH into $TMPD^{+\cdot}$, if the loss due to radical–radical reactions is taken into account.

As seen in Figure 2, the yield of $TMPD^{+\cdot}$ decreases with increasing pH to a minimum at pH 12, and at higher pH values the yield increases again to reach ≈80% of the value at pH 7. Above pH 12 the rate constant for reaction with TMPD of the cytosine-derived radical is 2×10^9 M^{-1} s^{-1}, i.e., equal to that measured in the *absence* of O_2, which suggests that at pH ≥12 TMPD is oxidized by the same radical as that in the absence of O_2.

2. Reaction Mechanism

2.1. Oxidizing Radicals Produced in the Absence of O_2 at pH 7–8. At pH 7 the rate constants for reaction of OH with cytosine[10] and TMPD[18] are 6.8×10^9 and 1×10^{10} M^{-1} s^{-1}. The latter reaction leads to the quantitative formation of $TMPD^{+\cdot}$.[18] On

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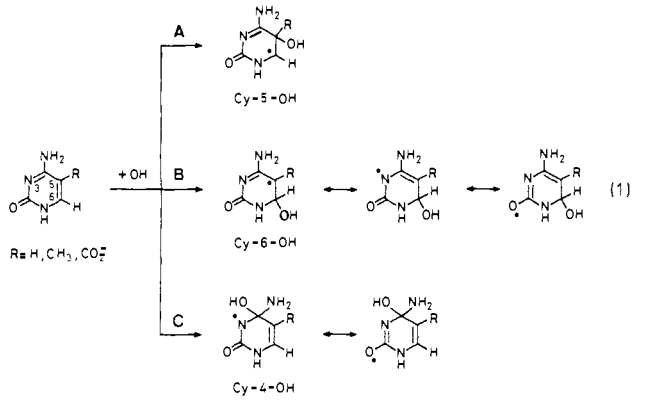
this basis, the fraction of $TMPD^{+\cdot}$ formed by direct reaction with OH is calculated to be 0.7/5.7 = 0.12 for solutions containing 2 mM cytosine and 0.2 mM TMPD. As seen from Figure 2, at pH 7–7.5 the experimentally observed yield (per OH) of $TMPD^{+\cdot}$ is 0.22, i.e., in addition to the $TMPD^{+\cdot}$ formed by direct reaction with OH, about the same amount is due to reaction of cytosine-derived radical(s) with TMPD. The rate constants for this and analogous reactions (Table II) are a factor 10–30 lower than that for reaction with OH.

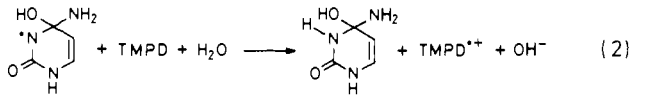
The results are explained by assuming that OH reacts with the cytosines by addition, as has been suggested earlier.[3-6,8-10,12] There are two sites for addition, N(3)=C(4) and C(5)=C(6), as shown in eq 1. Attachment of OH at C(5), C(6), and also at C(4) has



(1)

been taken into consideration, but it is much less likely to occur at N(3). This assumption is based on the electrophilic nature of OH, as a result of which the attack at an atom with an electron affinity comparable to that of oxygen is unfavorable.

The radicals formed by addition at C(4) and C(6), Cy-4-OH and Cy-6-OH, should have oxidizing properties, due to the unpaired spin density at the electron-affinic heteroatoms, as shown in eq 1B and C and in eq 2 and 3. Cy-4-OH, which contains



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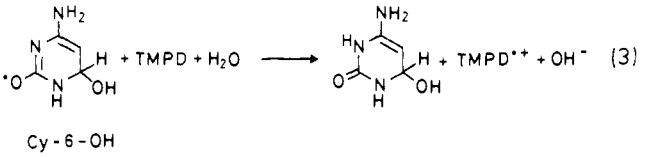

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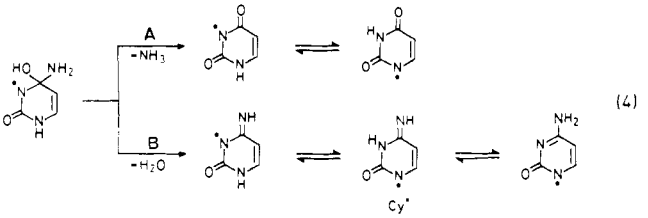
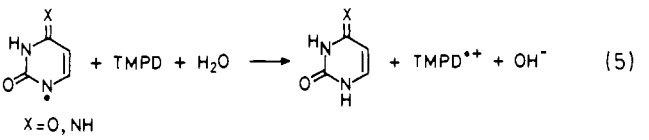


(4)

a semiaminal function, could undergo elimination of NH_3 (eq 4A) to yield the uracilyl radical, which can also be obtained[14,15] by oxidation of uracil. This radical has been shown to be able to oxidize TMPD.[14] However, the rate constant for reaction 4A has been estimated[12] to be $<50\ s^{-1}$, a value too low, under pulse radiolysis conditions, to enable reaction 4A to participate in the production of $TMPD^{+\cdot}$. Alternatively, elimination of H_2O from Cy-4-OH is possible (eq 4B). This would lead to Cy·, the same radical as that formed by dehydration of Cy-5-OH (see eq 7). Cy· is able to convert TMPD to $TMPD^{+\cdot}$ according to eq 5 (see section 2.3). The potential oxidizing radicals (eq 2, 3, and 5) produced by reaction of OH with cytosine are thus identified as Cy-6-OH and Cy-4-OH or its product according to eq 4B. With



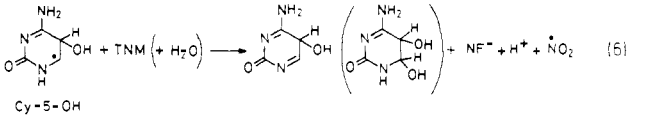
(5)

respect to being able to oxidize TMPD, Cy-6-OH is analogous to the radical formed by addition of OH to C(6) of uracil.[14]

As shown in Table II, the yield of $TMPD^{+\cdot}$ is enhanced on substitution at C(5), and it is decreased by substitution at C(6). This dependence on the pattern of substitution at C(5)/C(6) and the complementary one observed for the percentages of the reducing radicals (see Table III and section 1.2) indicates that Cy-6-OH is the main oxidizing radical, i.e., that Cy-4-OH, if formed at all, is of minor importance. The same conclusion is reached from a comparison of the percentages of oxidizing and reducing radicals from cytosine and *N*(1)-methylcytosine on the one hand and *N*(3)-methylcytosine on the other (Table II). The percentages are the same, although *N*(3)-methylcytosine does *not* contain a double bond between N(3) and C(4). These findings are in agreement with the (qualitative) results of previous studies, where it was concluded from kinetic[9] and conductivity[12] data that the main site of OH addition is at C(5)/C(6).[21]

2.2. Nature of the Reducing Radical. In Figure 2 it is shown that the yield (per OH) of $TMPD^{+\cdot}$ increases with pH to reach the 100% value (after correction for radical–radical decay; see section 1.1) at pH ~12. It is also seen that the yield of reducing radicals, as measured by the formation of nitroform anion, NF^-, decreases from 0.87 to <0.05 over the same pH range. Obviously, a reducing radical is transformed into an oxidizing one by reaction with OH^- (see section 2.3).

Of the radicals formed by addition of OH to cytosine (eq 1), only Cy-5-OH can be expected to have reducing properties. Cy-5-OH is of the α-aminoalkyl type, which have been shown to be powerful one-electron donors,[22,23] e.g., eq 6. This type of



(6)

reaction has also been observed with the radical formed by OH addition to C(5) of uracil.[14]

Hayon and Simic[9,22] have previously reported that reducing radicals are produced by reaction of OH with pyrimidines. Using menaquinone as oxidant for Cy-OH at pH 7, they observed the formation of the semiquinone with a yield of 60%, to be compared with the 87% yield obtained by using TNM (Figure 2). The lower number (60%) probably results from the very high dose rates used in the earlier[9] study, or it may be due to a contribution of a reaction other than electron transfer between Cy-5-OH and the quinone.

As seen in Table III, the yields of reduced TNM, as measured by the concentration of NF^-, depend on the position of the substituent at C(5)/C(6) in a way that is opposite to that observed with the yield of $TMPD^{+\cdot}$. This dependence on the pattern of substitution is expected if NF^- measures the concentration of Cy-5-OH. With the cytosine nucleosides and nucleotides the yields of reducing radicals are slightly (≤4%) larger than with cytosine or 1-methylcytosine. This small increase in $[NF^-]$ is probably due to the radicals produced by H abstraction from the (deoxy)ribosyl moiety. These radicals, except for those from C-2′ (with deoxy derivatives) and C-5′ (with the nucleotides), are expected[24,25] to be of the reducing type. On this basis the ratio of OH addition (at C(5)/C(6)) to H abstraction (at the (deoxy)ribose part) from nucleosides and nucleotides may be esti-

(21) Cy-4-OH could, in principle, yield uracil via eq 4A and 5 and, after elimination of NH_3 from reduced Cy-4-OH, via eq 2. However, after γ radiolysis the yield of uracil per OH radical was found by HPLC to be <0.5%.

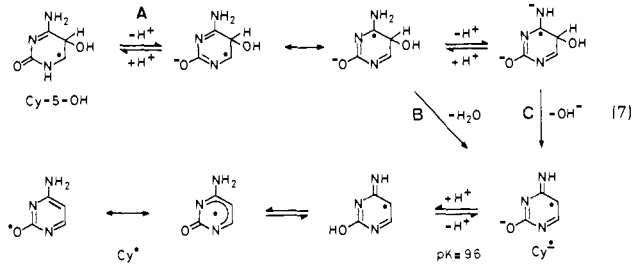
(22) Simic, M.; Hayon, E. *Int. J. Radiat. Biol.* **1972**, *22*, 507.
(23) Rao, P. S.; Hayon, E. *Biochim. Biophys. Acta* **1973**, *292*, 516.
(24) Asmus, K.-D.; Möckel, H.; Henglein, A. *J. Phys. Chem.* **1973**, *77*, 1218.
(25) Eibenberger, J.; Schulte-Frohlinde, D.; Steenken, S. *J. Phys. Chem.* **1980**, *84*, 704.

mated. A large proportion of H abstraction would not increase [NF^-] much, but it would decrease [$TMPD^{+}\cdot$] considerably. As seen from Table II, within experimental error the yield of [$TMPD^{+}\cdot$] does not decrease in going from cytosine to its nucleosides and nucleotides. In comparison, changing the substitution at C(5)/C(6) of the heterocyclic ring results in a pronounced change in [$TMPD^{+}\cdot$] (and [NF^-]; compare cytosine with cytidine, 5-methylcytosine, and 5-methylcytidine). It is therefore estimated, on the basis of the experimental error limits (±10%) of the yields, that only <20% of the OH radicals react with the cytosine nucleosides and nucleotides by H abstraction from the sugar moieties. This number is in agreement with estimates (20%) based on the rate constants[26] for reaction with the individual components of nucleosides and nucleotides.

Column 5 of Table III contains the sum of the yields per OH of Cy-5-OH and Cy-6-OH, determined at pH 6–8 via NF^- and $TMPD^{+}\cdot$. The values are equal to the yield of OH, except for the case of 5-carboxylcytosine, where the sum of the yields of Cy-5-OH and Cy-6-OH exceeds that of OH by 5–10%, and for 5-methylcytosine, where 12% is missing for a complete mass balance. However, with this compound the yield of $TMPD^{+}\cdot$ was 100% in the presence of O_2 (see section 1.3). This apparent discrepancy is resolved if it is assumed that 12% of the OH radicals abstract H from the methyl group and the resulting benzyl-type radical adds O_2 to give a peroxyl radical which oxidizes $TMPD^{+}\cdot$. A similar reaction has been observed in the case of thymine.[14]

It should be added that the $G(NF^-)$ values from Table III probably contain a contribution due to the H atoms produced by the radiation. If *all* the H atoms lead to NF^-, the correction leads to a reduction of the Cy-5-OH/OH and therefore the (Cy-5-OH + Cy-6-OH)/OH values by 10%. However, the values from Tables II and III refer to a dose rate of ≈200 rd/pulse. Under these conditions, a correction for the effect of radical–radical decay (which competes with the scavenging of Cy-5-OH and Cy-6-OH by TNM and TMPD, respectively) results in an increase of the values by 10%, thus compensating the apparent effect of the H atoms on the material balance.

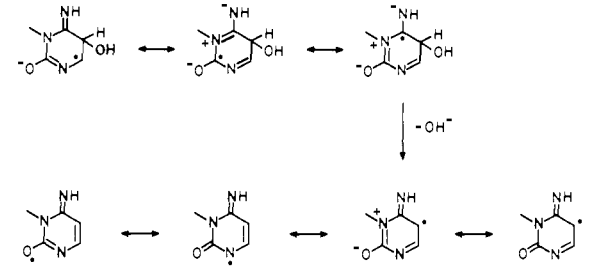
2.3. Conversion of Cy-5-OH in Alkaline Solution. As seen in Figure 2, the OH^--catalyzed reaction by which reducing radicals are converted into oxidizing radicals becomes visible at pH ≈8. An OH^--induced reaction of an OH adduct of cytosine has been described previously.[8,9,12] Meyers et al.[8] have attributed this reaction to ionization of Cy-5-OH or Cy-6-OH and Hayon and Simic[9] have interpreted it as due to ring opening of Cy-6-OH, whereas Hissung and von Sonntag[12] concluded that Cy-5-OH was in some way responsible. The ionization and the ring-opening hypotheses are excluded by the experimental data, which show that (a) the reducing radical Cy-5-OH is quantitatively transformed into an oxidizing one (upon ionization Cy-5-OH would become an even stronger reductant) and (b) Cy-6-OH (which is the only reasonable candidate for a ring-opening reaction) is formed with a yield much too low to account for the quantitative production of $TMPD^{+}\cdot$ at high pH. The finding (a) supports an earlier study,[11] where an OH^--induced change in the redox properties of a cytosine OH adduct was observed by pulse polarography. The formation of a stronger oxidant at high pH was reported. It is now suggested that this process involves dehydration of Cy-5-OH, as shown in eq 7. The dehydration steps 7B and



C are analogous to reactions observed with OH adducts of phenols,[27] of pyridones,[16] and of uracils.[14,15] The rates of dehydration, k_{deh}, of Cy-5-OH increase with increasing pH. The dependence of k_{deh} on pH does not follow a titration curve but may be described by the empirical relation $k_{deh} = 2.9 \times 10^6\ [OH^-]^{1/2}\ s^{-1}$ (Figure 1), which probably reflects the involvement of more than one dissociation and elimination reaction, cf. eq 7. In the case of the corresponding uracil-derived radical (U-5-OH) a similar relation was obeyed;[14] however, the slope of the k_{deh} vs. $[OH^-]^{1/2}$ plot was lower by the factor 3.8. This shows that Cy-5-OH undergoes dehydration more easily than U-5-OH, probably because the cytosine system is more electron-rich than the uracil system. As expected on the basis of the (heterolytic) elimination of OH^-, the susceptibility for dehydration increases on substituting H by methyl groups at C(5)/C(6). For instance, with 6-methylisocytosine the slope of the k_{deh} vs. $[OH^-]^{1/2}$ plot is 3.9×10^7, i.e., a factor >10 larger than with unsubstituted cytosine. In comparison, if C(5) is substituted by the (weakly) electron-withdrawing carboxyl group, the dehydration reaction becomes visible only at pH ≥11. Again, similar effects of substituents on the rates of dehydration have been observed previously with OH adducts of phenols,[28,29] of pyridones,[16] and of uracils.[14]

It may be noted that if the pH-dependent rates of dehydration are expressed in terms of an overall second-order rate constant for reaction of OH^- with Cy-5-OH and if the empirical formula given above is used, values between 1×10^8 and $3 \times 10^8\ M^{-1}\ s^{-1}$ are obtained for the pH range 10–11. Rate constants of this magnitude were reported by Meyers[8] and Hissung.[12]

The effect of substituents at N(1) is totally different from that at N(3). *N*(3)-Methylcytosine shows about the same susceptibility for OH^--induced dehydration as cytosine, whereas all the N-(1)-substituted cytosines do not at all undergo dehydration. Obviously, dehydration is possible only if the cytosine is ionizable at N(1)/C(2)-OH, as shown in eq 7A and 8. With *N*(3)-



methylcytosine an imino group is present. This does not seem to inhibit or slow down the dehydration reaction (eq 8). The absence of OH^--induced first-order changes with N(1)-substituted cytosines has been reported earlier.[8,9,12] The inability of N(1)-substituted Cy-5-OH radicals to undergo dehydration is of biological importance with respect to, e.g., the radiation chemistry of DNA. As a result of this inability to dehydrate, such a Cy-5-OH remains a reducing radical. The same type of behavior as that shown by N(1)-substituted Cy-5-OH radicals is also observed with the corresponding radicals from uracil and thymine.[30]

In order to confirm the identity of $Cy^{-}\cdot$, produced from Cy-5-OH by dehydration, $Cy^{-}\cdot$ was generated by reaction of cytosine with the oxidizing radical $SO_4^{-}\cdot$. As seen in Figure 3, the optical absorption spectrum B of the species from the reaction of cytosine with $SO_4^{-}\cdot$ at pH 11 resembles closely that (C) from the reaction of cytosine with OH at pH 13. This spectrum was measured after completion of the dehydration reaction, which, at pH 13, occurs in <10 μs. Spectra B and C can be made to quantitatively coincide in the 350–450-nm region by correcting for the contribution of Cy-6-OH, i.e., by taking into account that only Cy-5-OH but not

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(27) Land, E. J.; Ebert, M. *Trans. Faraday Soc.* **1967**, *63*, 1181. Adams, G. E.; Michael, B. D. *Ibid.* 1171.

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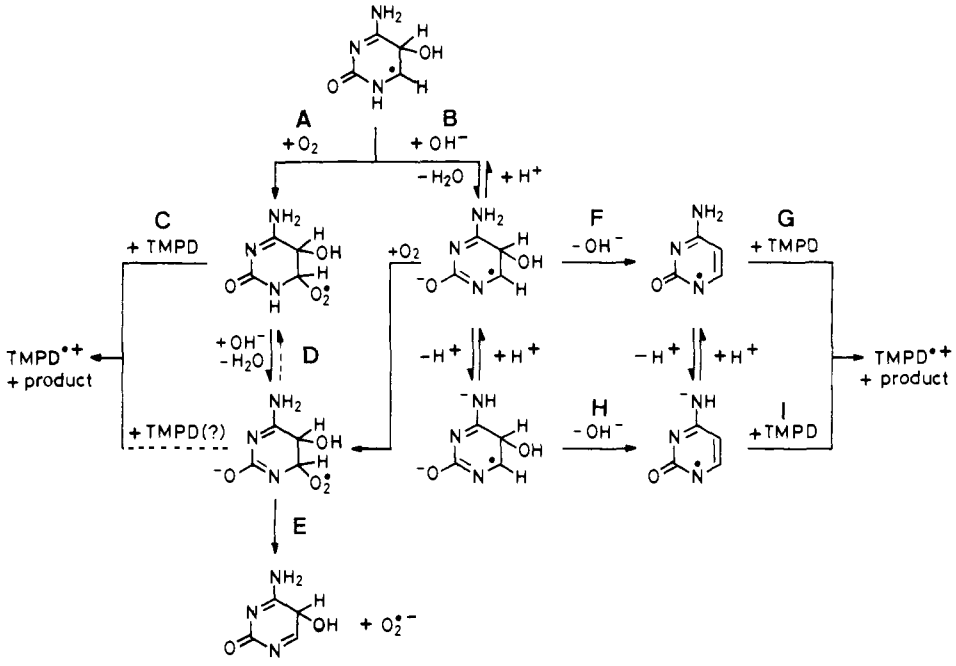
(30) Maruthamutu, P.; Steenken, S., unpublished material.

Scheme I



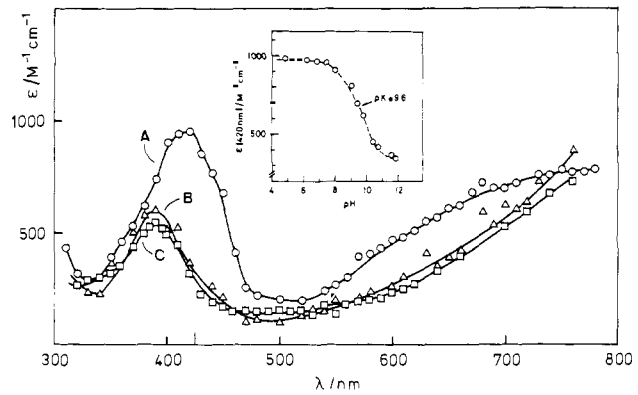
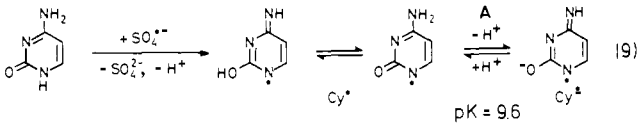


Figure 3. Absorption spectra of oxidized cytosine species: (A) ○, radical Cy•, measured 20 μs after reaction of e_{aq}^- with 60 mM $K_2S_2O_8$ in the presence of 60 mM *tert*-butyl alcohol and 0.8 mM cytosine at pH 6.6; (B) △, radical Cy^-•, measured under the same conditions at pH 11; (C) □, the radical Cy^-•, measured at 20 μs after reaction of OH with 0.5 mM cytosine at pH 13. This spectrum is *not* corrected for the contribution of Cy-6-OH (see text). The extinction coefficients are based on $G(e_{aq}^-) = G(SO_4^{-}\cdot) = G(Cy^-\cdot) = 3.6$ and $G(OH) = 6.3$. Inset: The dependence on pH of $\epsilon_{420\,nm}$ gives a pK_a of 9.6 for Cy•.

Cy-6-OH is able to undergo dehydration to yield Cy^-• (eq 7). For this purpose, [Cy-5-OH]/[Cy-6-OH] was taken as 0.9 (Tables II and III). The quantitative agreement of spectra B and C (except in the 450–550-nm range, where with C there is probably a spectral contribution due to Cy-6-OH) shows that Cy^-• is also produced by reaction with SO_4^{-}•, as shown in eq 9. The results



(9)

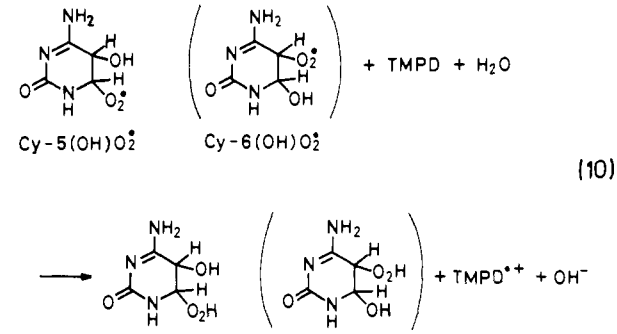
from the reactions with OH• and SO_4^{-}• thus mutually confirm the assignments of the radicals given in eq 1A and B (R = H) and in eq 7 and 9. The results also confirm the data from TMPD and TNM concerning the ratio Cy-5-OH/Cy-6-OH.

Spectrum A in Figure 3 is that from the reaction of SO_4^{-}• with cytosine at pH 6.6. It is suggested to represent Cy•, protonated Cy^-•. The pK_a value of Cy• (eq 9A) is 9.6, as seen from the inset in Figure 3. This value is very similar to that (9.7) determined[14] for the corresponding uracil-derived radical, which indicates that the electronic structures of the two radicals resemble each other closely.

The rate constant for reaction of SO_4^{-}• with cytosine was measured at pH 11 by monitoring the decay of SO_4^{-}•. The value is 7.5×10^8 M^{-1} s^{-1}, similar to that[15] for reaction with uracil.

Acid–base properties of an OH adduct of cytosine have been observed previously.[8,12] However, on the basis of the present results it is concluded that the reported pK_a values of 10.3[8] or 10.7[12] do not refer to a deprotonation equilibrium such as eq 7A but are "kinetic" pK values that reflect the OH^--induced dehydration reaction (eq 7A–C), which becomes clearly visible above pH 10.

2.4. Reactions of the Peroxyl Radicals Produced in the Presence of O_2. As described in section 1.3 the yield of $TMPD^+$• produced at pH 7–8 in the presence of O_2 represents quantitative conversion of the cytosine-derived radicals into $TMPD^+$•. Since oxygen is known[20] to react with organic radicals by addition and since peroxyl radicals oxidize TMPD to give $TMPD^+$•,[14] it is suggested that the production of $TMPD^+$• is due to the peroxyl radicals of Cy-5-OH (90%) and Cy-6-OH (10%); eq 10. The decrease in



(10)

the yield of $TMPD^+$• observed at pH >8 (Figure 2) is explained by base-catalyzed decomposition of Cy-5(OH)O_2• to yield O_2^{-}• and a nonradical (oxidized) cytosine derivative, as shown in Scheme I, reaction E. O_2^{-}• does not oxidize TMPD within ≤10 ms.[14] Reactions analogous to those in Scheme I were also observed with substituted uracils.[14]

At higher pH values the $TMPD^+$• yield does not decrease to ca. zero, as was observed[14] in the corresponding uracil case. This is explained by competition between O_2 and OH^- for Cy-5-OH (reactions A and B of Scheme I) and by competition between TMPD and OH^- for Cy-5-OH-O_2• (reactions C and D/E Scheme I). At low [OH^-] the reaction proceeds via steps A and C.

Between pH 10 and 12, steps D and E start to come in. Due to E, the $TMPD^{+\cdot}$ yield decreases. At pH ≥12 steps B, F, and H, by which $Cy^{-\cdot}$ is produced, become dominant, and the $TMPD^{+\cdot}$ yield increases again. The conclusion that, at pH ≥12, $Cy^{-\cdot}$ and not $Cy(OH)O_2\cdot$ is responsible for the oxidation of TMPD is supported by the observation that the rate constant for oxidation of TMPD (2×10^9 M^{-1} s^{-1}) is the same as that in the absence of O_2.

It may be interesting to note that, in contrast to the cytosine system, in the uracil system under the same conditions the dehydration reaction was not able to compete with the reaction with oxygen.[14] This again shows that Cy-5-OH dehydrates more easily than the analogous OH adduct of uracil, probably as a result of the higher electron density of cytosine as compared to uracil.

3. Conclusions

The reaction of the OH radical with cytosine and its derivatives has been shown to proceed by addition to the C(5)/C(6) double bond, with a ≈9:1 preference for addition at C(5). No evidence for addition at C(4)/N(3) was found. The radical formed by addition to C(5) has reducing properties, and that produced by attachment to C(6) is a weak oxidant. The radicals of the Cy-5-OH type undergo a base-catalyzed dehydration reaction to yield cytosine radicals that have oxidizing properties. Substituting H at N(1) by alkyl groups (as with the cytosine nucleosides and nucleotides) prevents the dehydration reaction.

From a comparison of the yields of oxidizing and reducing radicals from (a) cytosine and (b) cytosine nucleosides and nucleotides, it is estimated that >80% of the OH radicals react with the heterocyclic ring and not by H abstraction from the sugar part of the molecule.

In the presence of oxygen the OH adducts yield peroxyl type radicals which are able to oxidize TMPD. In basic solution these peroxyl radicals decompose, presumably by unimolecular elimination of $O_2^{-\cdot}$.

Registry No. OH, 3352-57-6; cytosine, 71-30-7; 3-methylcytosine, 19380-02-0; 5-methylcytosine, 554-01-8; 5-carboxylcytosine, 3650-93-9; 6-methylisocytosine, 3977-29-5; 1-methylcytosine, 1122-47-0; cytidine, 65-46-3; 2-deoxycytidine, 951-77-9; cytidylic acid, 63-37-6; 2-deoxycytidylic acid, 1032-65-1; 5-methylcytidine, 2140-61-6; 6-hydroxycytosine, 85761-81-5; 6-hydroxy-3-methylcytosine, 85761-88-2; 6-hydroxy-5-methylcytosine, 85761-84-8; 6-hydroxy-5-carboxylcytosine, 85761-82-6; 6-methyl-6-hydroxyisocytosine, 85761-87-1; 6-hydroxy-1-methylcytosine, 85761-89-3; 6-hydroxycytidine, 85761-91-7; 6-hydroxy-5′-deoxycytidine, 85761-90-6; 6-hydroxycytidylic acid, 85761-93-9; 6-hydroxy-2′-deoxycytidylic acid, 85761-92-8; 6-hydroxy-5-methylcytidine, 85761-80-4; 5-hydroxycytosine, 85761-80-4; 3-methyl-5-hydroxycytosine, 85761-95-1; 5-hydroxy-5-methylcytosine, 85761-85-9; 5-hydroxy-5-carboxylcytosine, 85761-83-7; 5-hydroxy-6-methylisocytosine, 85761-86-0; 5-hydroxy-1-methylcytosine, 85762-01-2; 5-hydroxycytidine, 85761-96-2; 5-hydroxy-2′-deoxycytidine, 85761-98-4; 5-hydroxycytidylic acid, 85761-99-5; 5-hydroxy-2′-deoxycytidylic acid, 85762-00-1; 5-hydroxy-5-methylcytidine, 85761-97-3.

Imidazole-Promoted Hydrolysis of Oxaphospholene Esters.[1] Nucleophilic vs. General Base Catalysis

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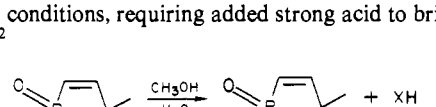
Abstract: A 2.4-fold excess of imidazole (Im) accelerated the hydrolysis of 5,5-dimethyl-2-methoxy-1,2-oxaphosphol-3-ene 2-oxide (**2a**) in 50% aqueous methanol by a rate factor of >50 compared to the uncatalyzed hydrolysis of **2a**. The reaction was first order in Im and **2a** and led to the imidazolium salt (**3**-Im) of 5,5-dimethyl-2-hydroxy-1,2-oxaphosphol-3-ene 2-oxide and methanol via two independent routes. One route involved direct nucleophilic attack at the methoxy carbon; the other route was a multistep process with nucleophilic attack at phosphorus to give a ring-opened intermediate which was observed by ^1H NMR. Im reacted slowly with **2a** in deuteriochloroform to give **3** and *N*-methylimidazole (**6**) via direct nucleophilic attack at the methoxy carbon. The stability of **6** and its conjugate acid in aqueous methanol precluded their intermediacy in the hydrolysis. Possible intermediate 5,5-dimethyl-2-imidazoyl-1,2-oxaphosphol-3-ene 2-oxide (**7**) was prepared and demonstrated to be highly reactive toward solvolysis in methanol and aqueous methanol. The Im-promoted hydrolysis was interpreted as involving general base catalysis.

We recently described the hydrolytic behavior of several esters and an amide of 5,5-dimethyl-2-hydroxy-1,2-oxaphosphol-3-ene 2-oxide (**1**). In 50% aqueous methanol the methyl (**2a**),[1,2] neopentyl (**2b**)[2] and phenyl (**2c**)[2] esters underwent hydrolysis to **1** without detectable intermediates, though in each case the instantaneous pseudo-first-order rate constant increased monotonically with time owing to acid catalysis provided by **1** (pK_a = 1.70 in water)[2]. The related *N,N*-diethylamide **2d** was nearly inert under these conditions, requiring added strong acid to bring about hydrolysis.[2]



2a, X = OCH_3
b, X = OCH_2-*t*-Bu
c, X = OPh
d, X = Et_2N

1

When methyl ester **2a** was treated with a slight excess of potassium hydroxide in aqueous methanol it was immediately converted to **3**, the conjugate base of **1**, again without detectable

(1) Paper 3 in the series Reactions of Oxaphospholenes. For paper 1, see: Macomber, R. S.; Krudy, G. A. *J. Org. Chem.* **1981**, *46*, 4038.
(2) Macomber, R. S.; Krudy, G. A.; Amer, M. Z. *J. Org. Chem.* **1983**, *48*, 1420.

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