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Pattern of OH Radical Reaction with Adenine and Its Nucleosides and Nucleotides. Characterization of Two Types of Isomeric OH Adduct and Their Unimolecular Transformation Reactions

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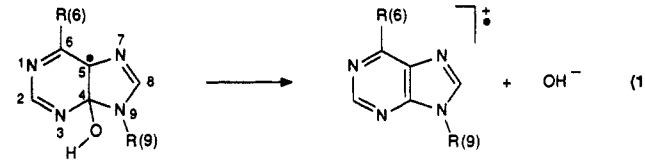
Abstract: The •OH radical reacts in aqueous solution with adenine, 9-methyladenine, adenosine and its 3′- and 5′-mono- and 3′,5′-diphosphates, and with the corresponding 2′-deoxyadenosines by addition to C4 and to C8 of the purine system. The resulting hydroxycyclohexa- or penta(aza)dienyl-type radicals A4OH• (formed by •OH addition at C4) and A8OH• (by addition at C8) undergo a dehydration and a ring-opening reaction, respectively, for which the activation parameters and pH dependencies have been determined. Dehydration of A4OH•, which yields an oxidizing N^6-centered neutral radical, is inhibited by H^+, whereas the ring opening of A8OH• is accelerated by H^+ (and also by OH^-). Product analysis data on adenine concerning the effect of additives on the yield of its depletion and of formation of 8-hydroxyadenine indicate that the N^6-centered radical is repaired by reduction by radicals derived from A8OH•.

Introduction

The DNA of the cell nucleus is known to be the most sensitive target for the radicals produced in living matter by, e.g., ionizing radiation.[3,4] Of the DNA constituents the pyrimidine and purine bases are more easily attacked than is the (aliphatic) deoxyribose backbone. It has long been suspected[3] but shown only recently[5] that radicals localized on bases can interact with ribose units to produce ribose radicals, which then[6] lead to strand breaks. In order to understand these processes it is necessary to identify the radicals produced by interaction with the bases of the highly reactive primary radicals from radiolysis of the predominantly aqueous biological systems. Of the primary species of aqueous-phase radiolysis, the •OH radical appears to be the most damaging.[3] Its reaction with *pyrimidines* is well understood: it consists essentially in addition to the C5/C6 double bond, with a pronounced preference for C5.[7] With the naturally occurring *purines*, however, a complete analysis of their reactions with •OH has so far not been possible,[8] although it has been shown,[9,10] by using a redox titration technique similar to that[7] applied in the case of the pyrimidines, that oxidizing and reducing radicals are formed.

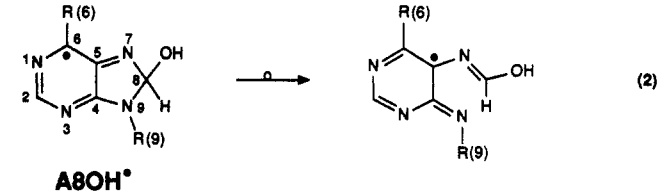
By use of fully alkylated adenines such as N^6,N^6-dimethyladenosine[11] or N^6,N^6,9-trimethyladenine[11,12] it has recently been possible to demonstrate by combination of conductance with optical detection techniques that a major part of the •OH radicals add at the C4/C5 double bond of the purine system to yield radicals that undergo unimolecular elimination of OH^-. This (heterolytic) dehydroxylation or dehydration reaction, which expresses itself by a *de*crease of optical density (OD) at 400 nm, leads to oxidizing radicals similar to those described[9,10] for the case of deoxyadenosine (phosphate). This process seems to be a general one for most 6- and 9-substituted purines, and substituent effects and activation parameters for eq 1 have recently been determined.[13]



(1)

A4OH•

An additional reaction that was found to be of universal occurrence on reaction of •OH with purines is a ring-opening reaction of the adduct formed by attachment of •OH to C8 of the purine system:[13]



(2)

A8OH•

This ring-opening reaction, which is characterized by an *in*crease of OD at ~330 nm, has long been inferred from product analysis studies.[8,14-16]

In the case of adenine [R(6) = NH_2] and its nucleosides and nucleotides, the room-temperature rates of the OD changes at ~300 and ~400 nm are accidentally the same or at least very similar,[13] giving the impression that there is only *one* transfor-

(1) Instituto Superior Tecnico.
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(3) For reviews see, e.g.: (a) *Effects of Ionizing Radiation on DNA*; Bertinchamps, A. J., Hüttermann, J., Köhnlein, W., Teoule, R., Eds.; Springer: Berlin, 1978. (b) Bernhard, W. A. *Adv. Radiat. Biol.* **1981**, *9*, 199. (c) von Sonntag, C. *The Chemical Basis of Radiation Biology*; Taylor and Francis: London, 1987.
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mation reaction. In order to prove that there are really *two* reactions it was necessary to vary in a systematic way parameters such as temperature, pH, and the nature of the (deoxy)ribosyl (phosphate) substituent at N9 and to study the effect of these on the transformation rates of the radicals (eqs 1 and 2). The result is that reactions 1 and 2 have different activation parameters and pH dependencies and can thereby be distinguished clearly from one another. In the transformation reactions are produced radicals with different redox properties, in agreement with earlier results,[13,17] and their intermolecular reactions are such that a portion of the radicals is repaired, leading to restoration of the starting material.

Experimental Section

Commercially available adenine derivatives were from Aldrich, Fluka, Merck, or Sigma and were used as received. *N,N,N',N'*-Tetramethyl-*p*-phenylenediamine (TMPD) was from Fluka, obtained as the dihydrochloride. The aqueous solutions (water purified with a Millipore Milli-Q system) typically contained 0.1–2 mM of the purine, and they were saturated with N_2O (to convert e_{aq}^- into •OH via $e_{aq}^- + N_2O + H_2O \rightarrow$ •OH + OH^- + N_2). Doses (from a 3 MeV van de Graaff accelerator, pulse width 400 ns) were chosen such that 1–2 μM radicals was produced, using N_2O-saturated 10 mM KSCN solutions for dosimetry and taking G(•OH) = 6.0 and $\epsilon((SCN)_2^{\bullet-})$ at 480 nm to be 7600 M^{-1} cm^{-1}.[18] The irradiated solutions were thermostated to ≤0.1 °C with cells that are an integral part of a heat exchanger.[19] Temperature variations were performed in the range 0–40 or 0–80 °C with 5–10 °C intervals using at least six temperatures. Arrhenius plots had correlation coefficients of ≥0.99.

For the product analysis studies, 8-hydroxyadenine (8-OH-A) and 5-formamido-4,6-diaminopyrimidine (FAPy) were prepared as standards, 8-OH-A (as the sulfate) from 4,5,6-triaminopyrimidine sulfate (Sigma) and phosgene, according to the method of Cavalieri and Bendich,[20a] and FAPy by reacting 4,5,6-triaminopyrimidine (free base obtained from the sulfate, see above) with formic acid, according to ref 20b. Aqueous solutions containing adenine and saturated with N_2O were ^{60}Co γ-irradiated. Analysis of the irradiated solutions was by HPLC with optical and electrochemical detection (glassy carbon electrode at +0.7 V). A 4.6 × 125 mm Nucleosil-5-C18 column was used with an aqueous 10 mM KH_2PO_4 solution containing 1% MeOH as the eluent. The (electrochemical) detection limit of 8-OH-A corresponded to 0.1 μM. The retention times of A and 8-OH-A were 17 and 16 min, respectively.

Results and Discussion

(a) Unimolecular Transformation Reactions and Their Dependence on Temperature. In Figure 1 are shown the absorption spectra of the transients produced on reaction of •OH with a series of adenine derivatives that differ with respect to the substituent at N9. The spectra observed on completion[21] of the reaction with •OH (recorded at 2–5 μs after the pulse) are obviously very similar, which shows that there is only a very small influence of the substituent at N9 on the shape of these spectra.

In all cases the spectra undergo time-dependent changes, characterized by *in*creases in optical density in the region ~320–350 nm and above ~520 nm, and by *de*creases at ~390–420 nm. The resulting spectra are also shown in Figure 1 (recorded 30–260 μs after the pulse). The similarity in the spectra from the different compounds indicates that the nature of the transients is similar, i.e., that the influence of the substituent at N9 on the kind and yields of the "transformed" radicals is small. Near room temperature, the changes at these wavelengths, which have been previously observed with some of the compounds,[13,17,23] have approximately the same rates. These rates are independent of the concentration of the substrates (between 0.1 and 2 mM), of the concentration of the initially produced radicals (between 0.5 and 5 μM), and of the pH (between 5 and 10; see later), but they are dependent on temperature. It is therefore concluded that the OD changes are due to unimolecular transformation reactions of the initially produced radicals, which are identified as •OH radical adducts to the adenine system, symbolized by AOH•. It was also found (for the case of the nucleosides) that the rates of the transformation reactions of the OH adducts are not changed by the addition at pH 5–8 of phosphate up to 20–40 mM, from which it can be calculated[24] that the rate constants for phosphate catalysis of the transformations are $<10^6$ M^{-1} s^{-1}. The radicals *produced* by the unimolecular transformations decay on the millisecond time scale (not shown) by what appears to be a bimolecular reaction, although slow (further) first-order components cannot be excluded.

In the case of OH adducts of purines with substituents at C6 other than NH_2, it has been demonstrated that the changes at ~330 nm and at ~400 nm are due to different radicals, i.e., to the radical produced by attachment to C4 (A4OH•), which undergoes a dehydroxylation or dehydration reaction (eq 1), and to that formed by OH addition to C8 (A8OH•), which undergoes opening of the imidazole ring (eq 2). The two types of reaction could be distinguished from one another by (a) the effect of substituents at C6 on their rates, (b) their pH dependencies, and (c) their activation parameters.[11,13] As mentioned above and as shown in Table I, in the case of purines substituted at C6 by NH_2, i.e., with adenine and its nucleosides and nucleotides, the room-temperature rates of the unimolecular reactions as observed at ~330 and ~400 nm are very similar, and this situation has been interpreted in terms of the occurrence of only *one* transformation reaction. Van Hemmen[23] suggested the ring-opening reaction of A8OH• in order to explain the formation of 5-formamidopyrimidines (FAPy's) as stable products from the reaction with •OH, whereas O'Neill[17] proposed a dehydration reaction of A4OH• to be responsible for the OD changes. This suggestion was based on the unimolecular formation of a radical with oxidizing properties.[17]

In order to test whether with adenine and its nucleosides and nucleotides there are also *two* rather than one transformation reactions, the rates of the OD changes at 330, 400, and 550–650 nm were studied at different temperatures in the range 0–80 °C. Figure 2 shows some of the results for adenosine as observed at 330 and 400 nm. Similar results were obtained for deoxyadenosine. Whereas the rates of the OD changes at 330 and 400 nm are exactly the same at 40 °C (see also Figure 3), the rates are different at the other temperatures. In particular, the relative order of the rates changes. Whereas at 0 °C the OD increase at 330 nm is faster than the decrease at 400 nm, at 70 °C the ratio of the rates is inverted, the decrease at 400 being now faster than the increase at 330 nm. Figure 3 is an Arrhenius plot of the data; it is evident that the OD changes are due to two different reactions characterized by different activation parameters (see Table I). The reaction represented by the OD *in*crease at 330 nm (ring opening of A8OH•; see below) has a low enthalpy and quite a negative entropy of activation, whereas for the OD *de*crease at 400 nm (dehydration of A4OH•) the values are higher. As seen from Figure 3, the two Arrhenius lines intersect (at ~40 °C; this is the temperature where the rates are exactly the same) and this is why the ratio of the rates inverts with temperature.

Concerning the influence of the substituents at N9, it is evident from Table I that the rates of both the OD buildup at 330 nm and the OD decrease at 400 nm decrease in going from the free base to the 2'-*deoxy*riboside to the riboside systems, and the rates of OD change are further decreased on introducing phosphate groups at C3' of the ribose or 2'-deoxyribose moiety. These phenomena are in agreement with the previously observed[13] rate-*de*creasing effects [via the inductive (-I) effect] of electron-withdrawing substituents at N9. As expected by the much shorter distance between the reaction site (the base radical) and C3' as compared to C5', the difference in the rates is much larger

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(19) Jagannadham, V.; Steenken, S. *J. Am. Chem. Soc.* **1984**, *106*, 6542.
(20) (a) Cavalieri, L. F.; Bendich, A. *J. Am. Chem. Soc.* **1950**, *72*, 2587. (b) Cavalieri, L. F.; Tinker, J. F.; Bendich, A. *Ibid.* **1949**, *71*, 533.
(21) At the concentrations of the purines (0.2–1 mM) and the rate constants for •OH reaction (~5×10^9 M^{-1} s^{-1}; see refs 8a and 22), •OH is scavenged in ≤1 μs.
(22) Buxton, G. V.; Greenstock, C. L.; Helman, W. P.; Ross, A. B. *J. Phys. Chem. Ref. Data* **1988**, *17*, 513.
(23) Van Hemmen, J. J. *Int. J. Radiat. Biol.* **1975**, *27*, 403.
(24) Assuming the average rate constant for transformation to be ~3×10^4 s^{-1}.

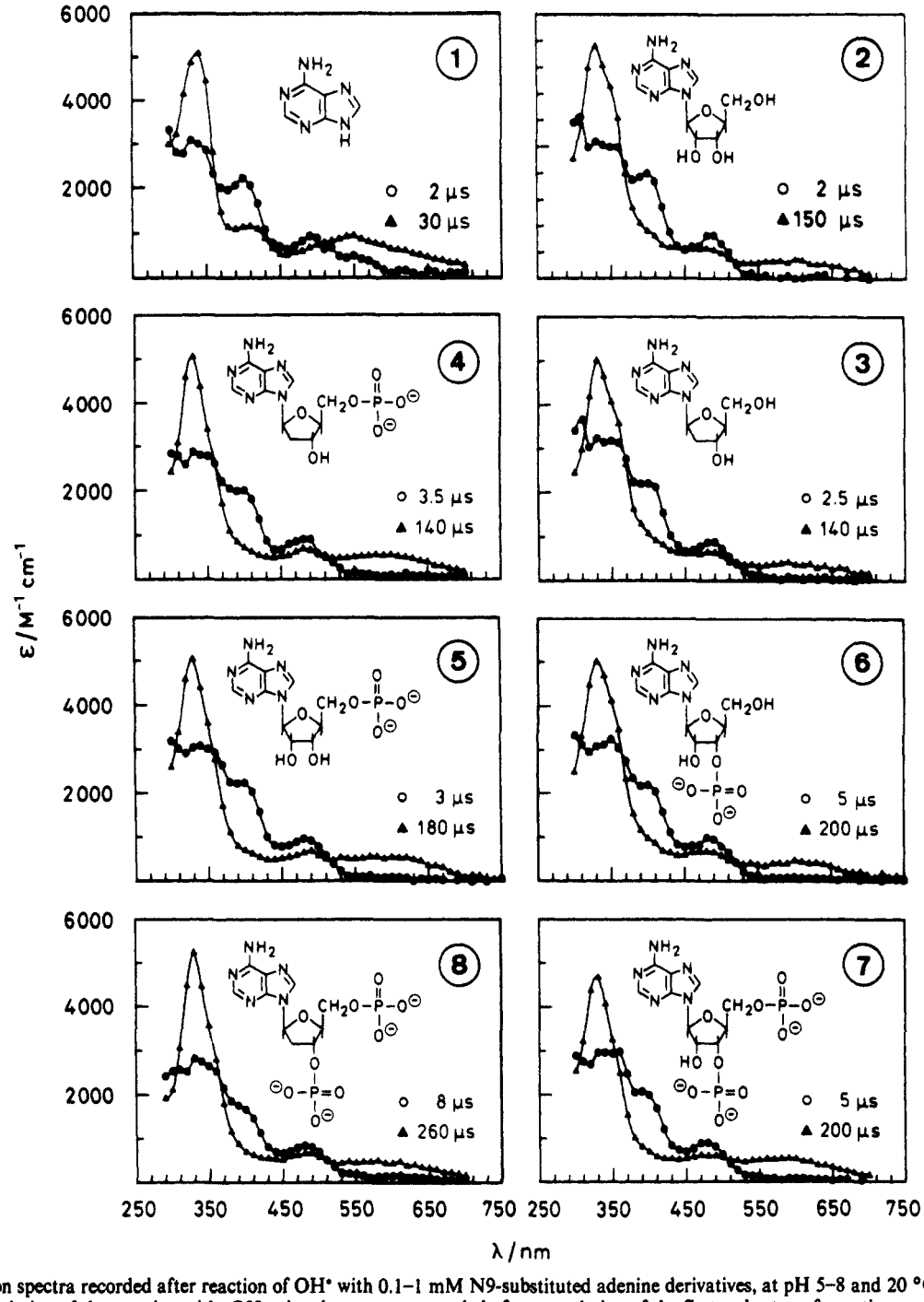


Figure 1. Absorption spectra recorded after reaction of OH• with 0.1–1 mM N9-substituted adenine derivatives, at pH 5–8 and 20 °C. Circles, spectra measured after completion of the reaction with •OH; triangles, spectra recorded after completion of the first-order transformation reactions. The parent compounds are identified by their formulas. The numbers refer to the entries in column 1 of Table I. The ϵ values are based on G(radical) = G(•OH) = 6.0, using $(SCN)_2^{\bullet-}$ as dosimetry and assuming $\epsilon((SCN)_2^{\bullet-})$ at 480 nn = 7600 M^{-1} cm^{-1}.

when the electron-withdrawing phosphate group is located at C3′ than at C5′. Inspection of the activation parameters (Table I) shows that the rate changes at 20 °C result from activation enthalpy *and* entropy changes.

Concerning the buildup at 550–560 nm, the rates and activation parameters are in-between those for the OD buildup at ~330 nm and the decrease at ~400 nm, indicating that the 550–650-nm OD changes are probably due to *both* A4OH• and A8OH• (see below).

(b) Identification of the Transformation Reactions. 1. Decay at 400 nm (Dehydration of A4OH•). In the case of N^6,N^6-dimethyladenosine[11] (DMAdo) and N^6,N^6,9-trimethyladenine[12] (TMA) it was shown by combination of optical with conductance measurements that the OD decrease at ~400 nm is due to dehydration of A4OH•. This type of reaction leads to radicals that are able to oxidize reductants such as ascorbate, thiol(ate)s, or *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD).[10,13,17] This kind of experiment was now also performed with adenine and adenosine 5′-phosphate, using TMPD as the scavenger for oxidizing radicals. The formation of one-electron-oxidized TMPD, i.e., $TMPD^{\bullet+}$, was observed. In both cases, the yield of $TMPD^{\bullet+}$ increased with increasing [TMPD] from 10 to 50 μM in an approximately linear fashion but reached a plateau at ~0.2 mM that extended to ~1 mM.

In the case of adenine, in order to obtain as precise a number as possible for the yield of oxidizing radical, to be used for a comparison with data from product analysis (see section d), a N_2O-saturated solution containing 4 mM adenine and only 0.1 mM TMPD (to avoid the direct reaction •OH + TMPD) was studied and a dose variation (between 50 and 500 rad/pulse) was performed. The yields of $TMPD^{\bullet+}$ increased with decreasing dose/pulse (due to decreasing radical/radical reactions that

Table I. Rate Constants and Activation Parameters[a] for the Transformation Reactions of the OH Adducts of 9-Substituted Adenine Derivatives in Aqueous Solution

compound[b] (temp range, °C)	p*K*	pH	k/s^{-1} (at 20 °C) buildup (nm)	k/s^{-1} (at 20 °C) decay (nm)	E_A/ kcal mol^{-1}	ΔS/ cal (mol K)$^{-1}$	log A/s^{-1}
adenine (**1**)	4.25, 9.83	7	1.3×10^5 (330)		7.4	−11.8	10.7
(0–70)			1.2×10^5 (550)		8.7	−7.4	11.6
				1.3×10^5 (400)	9.0	−6.5	11.8
				1.2×10^5[c]			
9-methyladenine	3.90	8.6–8.7	1.1×10^5 (330)		8.3	−9.3	11.3
(0–40)				1.1×10^5 (400)	8.9	−7.3	11.6
adenosine (**2**)	3.45, 12.5(S)[d]	7.3	2.6×10^4 (330)		8.2	−12.1	10.6
(0–80)			2.2×10^4 (550)		9.3	−8.4	11.4
			2.1×10^4 (620)		10.1	−6.1	11.9
				1.7×10^4 (400)	12.7	+2.3	13.7
adenosine 3′-monophosphate (**6**)	3.6–3.7	5	1.3×10^4 (330)		9.9	−7.9	11.5
(0–40)	5.92(P)[e]		1.4×10^4 (600)		11.9	−1.0	13.0
				1.1×10^4 (400)	12.7	+1.1	13.5
adenosine 5′-monophosphate (**5**)	3.7	5	2.1×10^4 (330)		8.7	−11.1	10.8
(0–40)	6.2–6.4(P)[e]		2.6×10^4 (580)		9.0	−9.7	11.1
				1.9×10^4 (400)	9.6	−8.3	11.4
adenosine 3′,5′-diphosphate (**7**)	~3.5	5	1.2×10^4 (320)		9.8	−8.6	10.3
(0–40)	~6(P)[e]		1.9×10^4 (580)		8.8	−10.9	11.8
				1.3×10^4 (390)	10.0	−7.5	11.6
2′-deoxyadenosine (**3**)	3.8	8.0	3.3×10^4 (330)		9.2	−8.3	11.4
(0–70)			2.6×10^4 (580)		9.2	−8.6	11.3
				2.0×10^4 (400)	11.8	−0.5	13.1
2′-deoxyadenosine 3′-monophosphate		5.2	2.3×10^4 (330)	1.4×10^4 (400)			
2′-deoxyadenosine 5′-monophosphate (**4**)	~3.7	5	2.7×10^4 (330)	2.4×10^4 (400)			
	~6.2(P)[e]		3.3×10^4 (580)				
2′-deoxyadenosine 3′,5′-diphosphate (**8**)	~3.5	5	1.7×10^4 (330)		9.7	−7.9	11.5
(0–70)	~6(P)[e]		2.5×10^4 (580)		8.8	−10.2	11.0
				2.2×10^4 (400)	10.7	−4.1	12.3

[a] The error in the E_A values is estimated to be ±10%; in the $\Delta S^{\ddagger}$ values, it is ±15%. [b] The number identifies the compounds in Figure 1. [c] Rate constant for formation of A(−H)•, obtained from the [TMPD]-independent rate of $TMPD^{\bullet+}$ formation; see Figure 4a. [d] The pK_a is assumed to relate to dissociation of C1′–OH. [e] Second pK_a of the phosphate group.

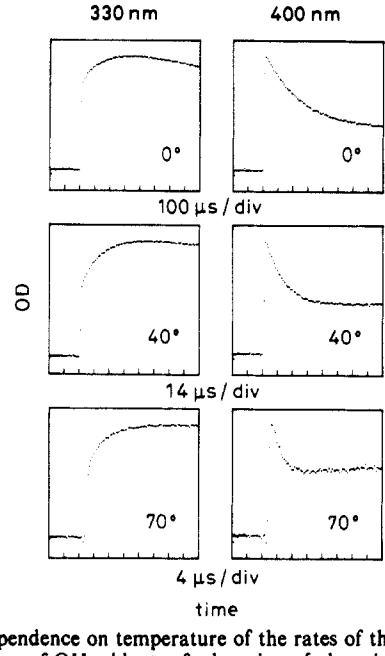


Figure 2. Dependence on temperature of the rates of the OD changes after production of OH adducts of adenosine. [adenosine] = 0.4 mM, [N_2O] = 20 mM, pH 7.5. The wavelengths of observation and temperatures are as indicated.

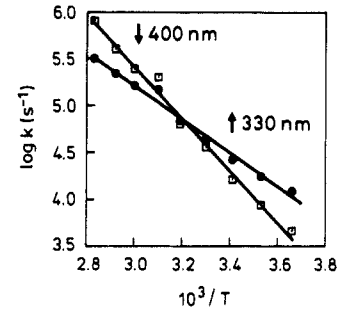


Figure 3. Arrhenius plots of the transformation reactions of the adenosine OH adducts (0.4 mM adenosine solutions, N_2O saturated, pH 7.5). Circles, OD buildup at 330 nm; squares, OD decay at 400 nm.

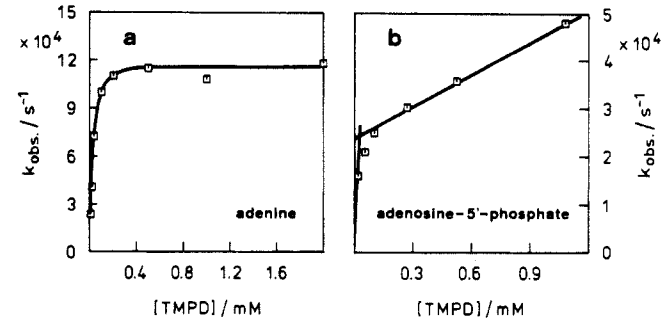


Figure 4. Dependence on [TMPD] of k_{obs} for the formation of $TMPD^{\bullet+}$ in N_2O-saturated 1–6 mM solutions of (a) adenine and (b) adenosine 5′-phosphate at 20 °C and pH 8.

prevent TMPD from being oxidized). Extrapolation to zero dose[25] and correction for the competition between adenine and N_2O for e_{aq}^- (by which the yield of •OH is reduced) gave a yield of 81% for the oxidizing radical (A(−H)•) from the •OH reaction with adenine.[26]

The effect of [TMPD] on the *rate* of $TMPD^{\bullet+}$ formation was also studied. The data obtained for adenine and adenosine 5′-phosphate are presented in Figure 4a and b. In the case of adenine (Figure 4a), k_{obsd} for $TMPD^{\bullet+}$ formation increases linearly with [TMPD] up to a plateau starting at ~0.2 mM. From the linear portion, the bimolecular rate constant for oxidation of TMPD by the oxidizing radical A(−H)• is obtained as 1.8×10^9 M^{-1} s^{-1}. The

(25) Steenken, S.; O'Neill, P. *J. Phys. Chem.* **1977**, *81*, 505.
(26) In support of this result is the observation, with time-resolved electrochemical detection, that •OH reaction with adenine leads with a high yield to an oxidizing radical: Busi, F.; Concialini, V.; Tubertini, O.; D'Angelotonio, M. *Radiat. Phys. Chem.* **1989**, *34*, 857.

plateau value is 1.2×10^5 s^{-1}. This number is essentially the same as the rate constant for the decrease of OD at ~400 nm (1.3×10^5 s^{-1}), which is assigned to dehydration of A4OH•. Therefore, the plateau in the k_{obsd} vs [TMPD] plot is explained in terms of dehydration of A4OH• being the rate-limiting step in the formation of TMPD•+, cf. eq 3a.

$$\text{A4OH}^\bullet \xrightarrow[-H_2O]{a\ k_{deh}} \text{A(-H)}^\bullet \xrightarrow[+H_2O]{b\ +\text{TMPD}} \text{A} + \text{TMPD}^{\bullet+} + \text{OH}^- \quad (3)$$

The situation is different in the case of adenosine 5′-phosphate, as seen from Figure 4b. Specifically, there is no plateau. The k_{obsd} vs [TMPD] dependence can be approximated by two individual lines. The slopes are interpreted to correspond to the bimolecular rate constants for oxidation of TMPD by one apparently strongly and one weakly oxidizing radical. The former is identified as A(-H)•, and the latter as its precursor, A4OH• (see eq 4). At low [TMPD], the rate of dehydration of A4OH•

$$\begin{array}{ccc} \text{A4OH}^\bullet & \xrightarrow[k = 1.9 \times 10^4\ \text{s}^{-1}]{-H_2O} & \text{A(-H)}^\bullet \\ \text{TMPD} \downarrow\ k = 2.3 \times 10^7\ \text{M}^{-1}\text{s}^{-1} & & \text{TMPD} \downarrow\ k = 2 \times 10^9\ \text{M}^{-1}\text{s}^{-1} \\ \text{TMPD}^{+\bullet} & & \text{TMPD}^{+\bullet} \end{array} \quad (4)$$

is fast as compared to the reduction of A(-H)• by TMPD, so it is A(-H)• which reacts with TMPD ($k = 2 \times 10^9$ M^{-1} s^{-1}).[27] At high [TMPD], however, A4OH• is scavenged by TMPD ($k = 2.3 \times 10^7$ M^{-1} s^{-1}) before it gets a chance to dehydrate.[28]

From the large difference in the rate constants for oxidation of TMPD by A4OH• and A(-H)•, it is obvious that the latter is the stronger oxidant. Increases in oxidation strength upon dehydration of OH adducts, α,β-dihydroxyalkyl radicals, and even nonradical systems are well-known.[29] With radicals, the enhancement of oxidizing power is probably due to the increase in unpaired spin density at the (electrophilic) hetero atoms (see eq 5, Scheme I). The ability of the precursor to A(-H)• to oxidize TMPD is in agreement with it being A4OH• rather than A5OH•, which—on the basis of the distribution of the unpaired electron—should be a strongly *reducing* radical.[11]

The larger oxidizing strength of the A4OH• of adenosine 5′-phosphate and of 2′-deoxyadenosine compared to that of adenine[30] is due to electron withdrawal by the ribose phosphate group at N9. The same effect causes the rates of the heterolytic dehydration reactions of A4OH• to decrease as compared to the adenine system (see Table I).

In the case of the •OH reaction with DMAdo, about 20% (relative to •OH) of the oxidizing radical of the A(-H)• type was formed, not by dehydration of A4OH• but of the isomeric A5OH•, produced by addition of •OH to C5 of DMAdo. A4OH• and A5OH• were distinguished from one another by the fact that the latter was *rapidly* scavenged by O_2, which leads to a corresponding reduction in the yield of TMPD•+,[11] whereas the former had a *low* reactivity. An analogous experiment was now performed with adenosine (Ado), with N^6-methyladenosine, and also with adenine. For this purpose, the yield of TMPD•+ was first determined in a N_2O-saturated solution at pH ~8 containing 2 mM of the base or the nucleoside (to scavenge the •OH radicals) and 0.2–0.4 mM TMPD (to scavenge the oxidizing radicals derived from the nucleoside). The N_2O was then replaced by a 4:1 mixture of N_2O and O_2, where the N_2O concentration is still sufficient to scavenge all the e_{aq}^- from the radiolysis of the water and there is enough O_2 to intercept any reducing organic radicals. It turned out that in the case of Ado the introduction of O_2 into the solution did *not* reduce the yield of TMPD•+, measured at 150 μs after the pulse, i.e., after completion of the reaction of TMPD with A(-H)• and *before* reaction of any of the peroxyl radicals formed[31] under these conditions. From this it is concluded that the tendency of the •OH radical to add to C5 of Ado to yield the reducing A5OH• (which is expected to react with O_2 with $k \geq 5 \times 10^9$ M^{-1} s^{-1})[11] is ≤5%. This is less than the 20% observed[11] in the case of DMAdo and TMA.[12] It thus appears that methylation of N^6 changes the selectivity of the •OH addition to the C4–C5 double bond of the adenosine system in favor of C5.[32] In agreement with this concept, with N^6-(mono)methyladenosine the yield of A5OH•, as determined by the O_2 effect on the yield of TMPD•+, was found to be ~10–15%, i.e., intermediate between those of the di- and the nonmethylated systems. In the case of adenine, the yield of "O_2-scavengeable" oxidizing radicals was 16% (assigned to A5OH•), compared to 65% "nonscavengeable" (identified as A4OH•) (see, however, below).

2. Increase at 330 nm (Ring Opening of A8OH•). The unimolecular increase in OD at ~330 nm of OH adducts of 9-substituted purine derivatives has previously been assigned[11,13] to the ring-opening reaction of the OH adduct to C8, A8OH•. One criterion was that this (reducing) radical could be more easily scavenged by oxidants such as O_2, as compared to the (oxidizing) A4OH•. The rate constant for reaction of O_2 with A4OH•, k_{O_2}(A4OH•), can be directly measured by the effect of O_2 on the decay kinetics at 400 nm, $k_{obsd} = k_{deh} + k_{O_2}[O_2]$, from which k_{O_2} was obtained as 1.0×10^9 M^{-1} s^{-1} for the case of deoxyadenosine. The corresponding rate constant for A8OH•, k_{O_2}(A8OH•), was determined via the quenching effect of O_2 on the yield of the OD buildup at 330 nm. At $[O_2] = 20$–30 μM, the OD buildup was inhibited by 50%, and if it is assumed that at this concentration ring opening ($k_{r.o.} = 1 \times 10^5$ s^{-1}) and scavenging by O_2 are equally efficient, k_{O_2}(A8OH•) results as $(4 \pm 1) \times 10^9$ M^{-1} s^{-1}. This larger rate constant is in agreement with the larger spin density on *carbons* expected for A8OH•, compared to A4OH•, in agreement with results obtained for DMAdo[11] and TMA.[12] However, the difference in the reactivities with O_2 between A4OH• and A8OH• is not very large, which means that O_2 is not a very good reactant to differentiate between the two isomeric radicals.

A different oxidant was therefore studied. Methylviologen (MV^{2+}) has been found to selectively oxidize A8OH• from DMAdo.[11] However, it turned out that in the case of Ado and 2′-deoxyadenosine the reactivity of MV^{2+} was too low. In the case of adenine, on the other hand, a rapid reduction of MV^{2+} by an adenine-derived radical was seen, which yielded the radical cation $MV^{\bullet+}$ with its characteristic absorption at 605 nm. However, there was no well-defined plateau in the $[MV^{\bullet+}]$ vs $[MV^{2+}]$ dependence, so the yield, at pH 6–7, of reducing radicals can only be roughly estimated as 15 ± 5% of the initial •OH concentration. The oxidant $C(NO_2)_4$ (TNM) was also investigated, using conductance detection at pH ~5 to measure the formation of H^+, derived from the oxidation by TNM of the radicals. No well-defined plateau in the $[H^+]$ vs [TNM] plot was seen, indicating that TNM, like MV^{2+}, is *not* sufficiently selective to differentiate between the isomeric radicals. At ~0.2 mM TNM, the yield per •OH of H^+ formed was ~30%.

(27) The rate constant was obtained by computer-fitting the k_{obsd} vs [TMPD] relation. The earlier[13] value for reaction of A(-H)• (5.2×10^9) is hereby withdrawn.

(28) Similar observations were made with 2′-deoxyadenosine, where k(A(-H)• + TMPD) = 2×10^9 and k(A4OH• + TMPD) ≈ 1×10^7 M^{-1} s^{-1}.

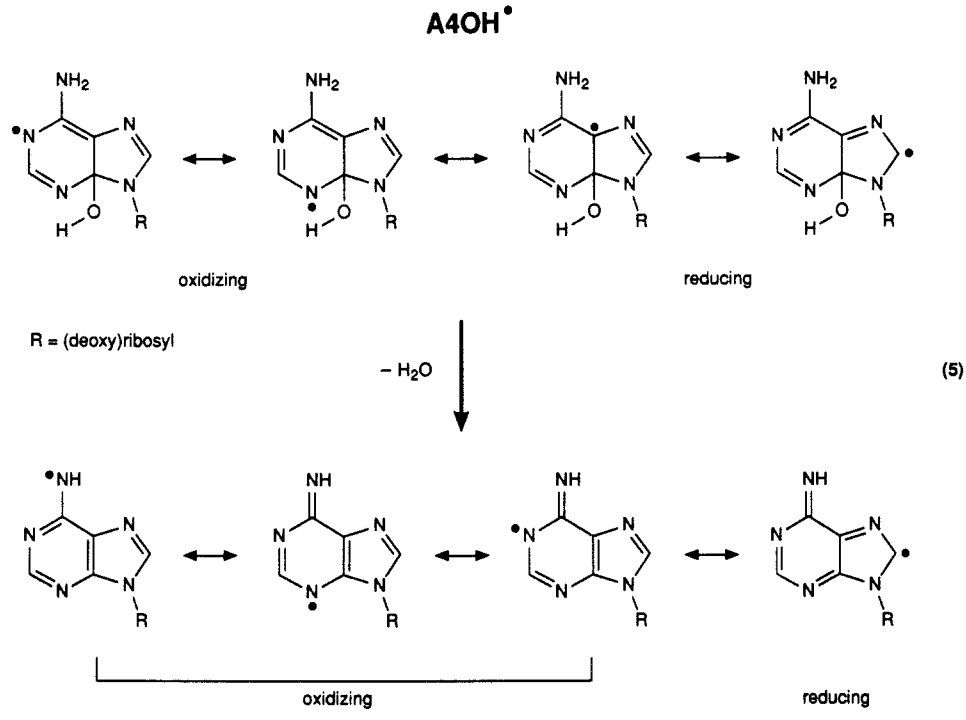
(29) Cf.; Steenken, S. *J. Chem. Soc., Faraday Trans. 1* **1987**, *83*, 113. Steenken, S. In *Free Radicals in Synthesis and Biology*; NATO ASI Series C260; Minisci, F., Ed.; Kluwer Academic: Dordrecht, The Netherlands, 1989; p 213.

(30) From the data in Figure 4a, it is estimated that for adenine k(A4OH• + TMPD) ≤ 5×10^6 M^{-1} s^{-1}.

(31) For rate constants for reaction of peroxyl radicals with reductants such as TMPD see: Neta, P.; Huie, R. E.; Mosseri, S.; Shastri, L. V.; Mittal, J. P.; Maruthamuthu, P.; Steenken, S. *J. Phys. Chem.* **1989**, *93*, 4099. Neta, P.; Huie, R. E.; Maruthamuthu, P.; Steenken, S. *Ibid.* 7654.

(32) Electron donation by substituents at C6 increases the electron density at C5 (≡ ortho) and not at C4 (≡ meta). The probability of attachment of the electrophilic •OH at C5 is thereby increased. From a steric hindrance point of view, one would expect the opposite effect. That the steric effect is not important is probably due to the small size of •OH.

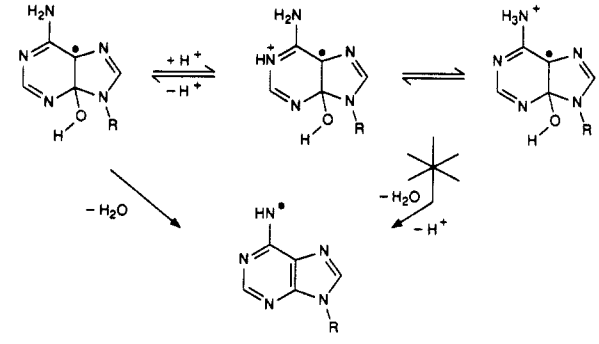
Scheme I



With the model compounds,[11,13] the ring-opening reaction of A8OH• was characterized by quite negative activation entropies, i.e., −21 cal (mol K)$^{-1}$. With the naturally occurring adenines, the entropies of the OD increases at 330 nm are also quite negative, ~−10 cal (mol K)$^{-1}$ (see Table I), which supports their assignment as due to ring opening of A8OH•.

(c) pH Dependence of the Dehydration and Ring-Opening Reactions. For the case of adenosine and deoxyadenosine, the rate constants for the OD *de*crease at ~400 nm (dehydration of A4OH•, eq 1), and for the OD *in*crease at ~330 nm (ring opening of A8OH•, eq 2) were measured at 0 °C to be able to differentiate better between the two processes. The rate constants have a different pH profile (see Figure 5 for the case of adenosine).[33,34] In both cases there is a broad range where the rate constants are independent of pH. However, starting at pH ~5, the rate constant for dehydration of A4OH•, k_{deh}, decreases to values of $<10^3$ s^{-1} as the pH is lowered, whereas the rate of ring opening of A8OH•, $k_{r.o.}$, more than doubles. At high pH, the rate of transformation of A4OH• is independent of pH (up to pH ~11), whereas the rate of ring opening of A8OH• increases by a factor of ~5 on going to pH ~11.5.

1. A4OH•. As in the case of N^6,N^6-dimethyladenosine,[11] the H^+-induced suppression of the dehydration of A4OH• can easily be explained by the decrease of the electron density of the radical upon protonation of N1 or of N^6 (by which the electron-donating NH_2 is converted to the electron-withdrawing NH_3^+, eq 6). In



(6)

a reaction such as heterolytic elimination with OH^- as the anionic leaving group,[35] the rate will be drastically reduced on decreasing the electron density of the molecule (as by protonation).

In the case of N^6,N^6-dimethyladenosine, dehydration of A4OH• was inhibited not only by H^+ but also by OH^-.[11] This effect was explained in terms of deprotonation from the OH group at C4 of A4OH• to give A4O•$^-$ (which cannot eliminate OH^-). In contrast, with adenosine, where N^6 is not methylated, OH^--induced deprotonation can also occur at N^6, resulting in the extremely strongly electron donating substituent NH^-, which would, if formed, enhance OH^- elimination from C4 enormously. Experimentally, there is neither an increase nor a decrease of the rate of A4OH• decay up to pH ~11 (see Figure 5), so it is concluded that the OH^--induced inhibition of OH^- elimination (by deprotonation from the OH group at C4) is just about canceled by the OH^--induced enhancement (by deprotonation from the NH_2 group at C6). The absence of inhibition by OH^- of dehydration is supported by the observation that the yield of TMPD•$^+$

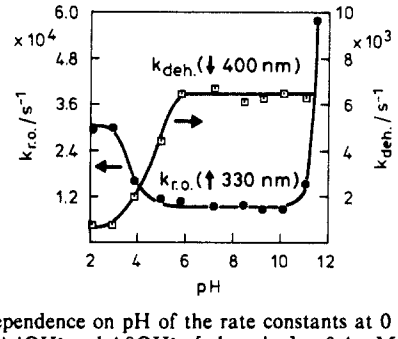


Figure 5. Dependence on pH of the rate constants at 0 °C for transformation of A4OH• and A8OH•. [adenosine] = 0.4 mM. Circles, OD buildup at 330 nm (ring opening of A8OH•); squares, OD decay at 400 nm (dehydration of A4OH•).

(33) A very similar dependence was found for deoxyadenosine.

(34) With the nucleo*tides*, there is a flat maximum in $k_{r.o.}$ and k_{deh} at pH 5–6. Above pH ~6 the rate constants decrease by ≤30% to reach constant values between pH 7 and 10. Activation parameter measurements (see Table I) were performed at pH 5 to have the phosphate group present in the monoanion form (as in DNA).

(35) OH^- elimination may also be concerted with deprotonation from the H_2N group, but this is considered less likely.

Scheme II. Mechanism of Acid- and Base-Catalyzed Ring Opening

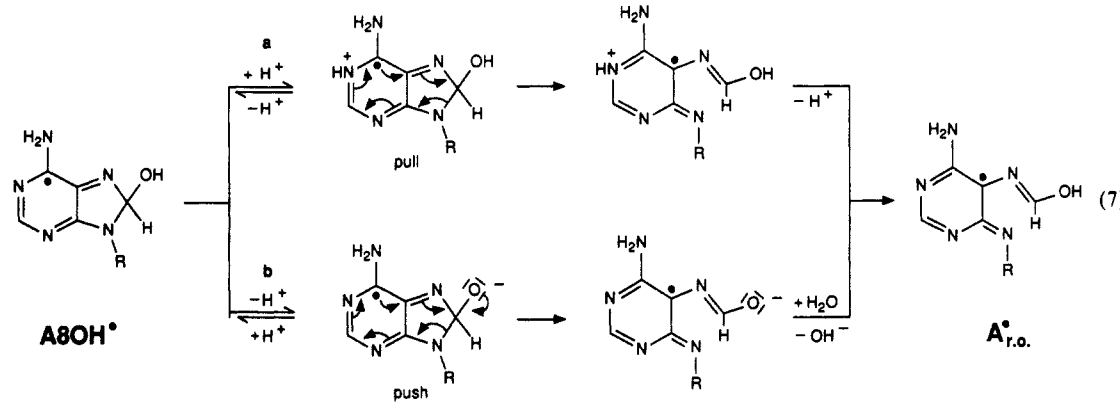


Table II. Yields[a] of Products and of Oxidizing and Reducing Radicals Produced in the Reaction of •OH with Adenine and Deoxyadenosine in Aqueous Solution at 20 °C. Redox Reactivity of the Radicals

purine	additive	loss of adenine[b]	8-OH-A	FAPy	TMPD•+ (A(−H)•)	$C(NO_2)_3^-$	$MV^{•+}$
adenine (A)		~30	5	2	81[c,d]	~30[e,f]	~15[f]
	0.2 mM O_2		8		65[h]		
	0.1 mM ascorbate		0.9[g]				
	0.2–2 mM $Fe(CN)_6^{3-}$	60	18	<0.2			
	k(A(−H)• + TMPD) = 1.8 × 10^9 M^{-1} s^{-1}						
2′-deoxyadenosine (dAdo)	k(A4OH• + O_2) = 1.0 × 10^9 M^{-1} s^{-1}[i]				32[i]	~50[i]	
	k(A8OH• + O_2) = (4 ± 1) × 10^9 M^{-1} s^{-1}[k]						

[a] In percent of •OH, assuming G(•OH) = 6.0. All solutions ([A] = 0.2–1 mM) were saturated with N_2O (~20 mM). The dose rate was 4.9 krad/min. Unless otherwise indicated, the pH was 6–7. [b] In 0.1 mM adenine solutions, at natural pH (~6); conversion 3–20%. The values are corrected for the contribution of •H to the loss of adenine assuming $G(H^•)$ = 0.6. [c] At pH ~8–9. [d] [A] = 4 mM, [TMPD] = 0.1 mM; value extrapolated to zero dose and corrected for the reaction of e_{aq}^- with adenine. [e] From conductivity measurements at pH 5 assigning the conductivity increase to the formation of NF^- and H^+ [from the oxidized radical(s)]. [f] In the dependence on [scavenger] of the yield of scavenged radical(s), a plateau is not well defined. [g] pH 7, 1 mM adenine. [h] Due to reaction of A(−H)• from A4OH• (see text). [i] From the O_2-induced increase in the rate of OD decrease at 400 nm. [k] From the O_2-induced inhibition of the OD increase at 330 nm and by analyzing the competition between ring opening and scavenging of A8OH•. [l] From ref 17.

[from A(−H)•] is *in*dependent of pH in the range 8–~11.5, in contrast to the case of N^6,N^6-dimethyladenosine, where it decreased above pH ~10.5, due to the base-induced inhibition of dehydration of A4OH•.[11]

2. A8OH•. In the case of the OD buildup at 330 nm, assigned to ring opening of A8OH•, there is an increase in rate not only below pH 5 but also above pH 10 (Figure 5). The H^+ catalysis is explained by the electron *pull* exerted by the pyrimidine ring on protonation of N1 (or N^6) (step 7a, Scheme II), leading to an increase in the heterolysis rate of the C8–N9 bond. Base catalysis of ring opening is suggested to proceed by deprotonation of A8OH• (step 7b) by which is produced sufficient electron density to "push out" the electron pair joining C8 and N9. These reactions are in agreement with the hemiamidal nature of A8OH•. These "tetrahedral intermediate" type compounds are expected to be sensitive toward H^+- and OH^--catalyzed decomposition.[36]

(d) Product Analysis Studies. Aqueous solutions containing 0.1–1 mM adenine, 0–1 mM $Fe(CN)_6^{3-}$, and 1 mM phosphate (pH 7) and saturated with N_2O were ^{60}Co γ-irradiated with doses such that adenine conversion never exceeded 15%, assuming G(−adenine) = 6.6. The irradiated solutions were analyzed for loss of adenine, and for formation of 8-hydroxyadenine (8-OH-A) and 5-formamido-4,6-diaminopyrimidine (FAPy), using HPLC with optical and electrochemical detection. The results are presented in Table II and in Figure 6, which shows the yield of 8-OH-A as a function of pH [at 0.8 mM $Fe(CN)_6^{3-}$] and of [$Fe(CN)_6^{3-}$] (at pH 7). In the absence of $Fe(CN)_6^{3-}$ as oxidant for hydroxycyclohexadienyl-type radicals ("OH adducts"),[37] both

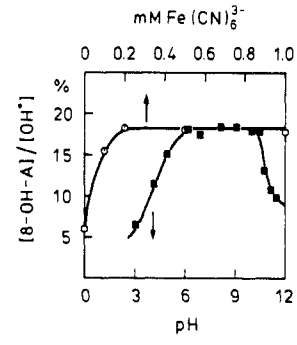


Figure 6. Dependence of the yield of 8-OH-A (as percentage of •OH) on (a) [$Fe(CN)_6^{3-}$] at pH 7 (circles) and (b) on pH in the presence of 0.8 mM $Fe(CN)_6^{3-}$ (squares).

8-OH-A and FAPy are formed (Table II). Addition of the oxidant to the system leads to an increase in the yield of 8-OH-A and to a decrease in that of FAPy. At ~0.2 mM $Fe(CN)_6^{3-}$, a plateau in the yield of 8-OH-A is reached (Figure 6), which corresponds to 18% of the •OH radicals initially present, while FAPy is no more detectible (Table II). The yield of 18% for 8-OH-A is in good agreement with an earlier estimate (20%) obtained[38] by conventional chromatographic techniques.

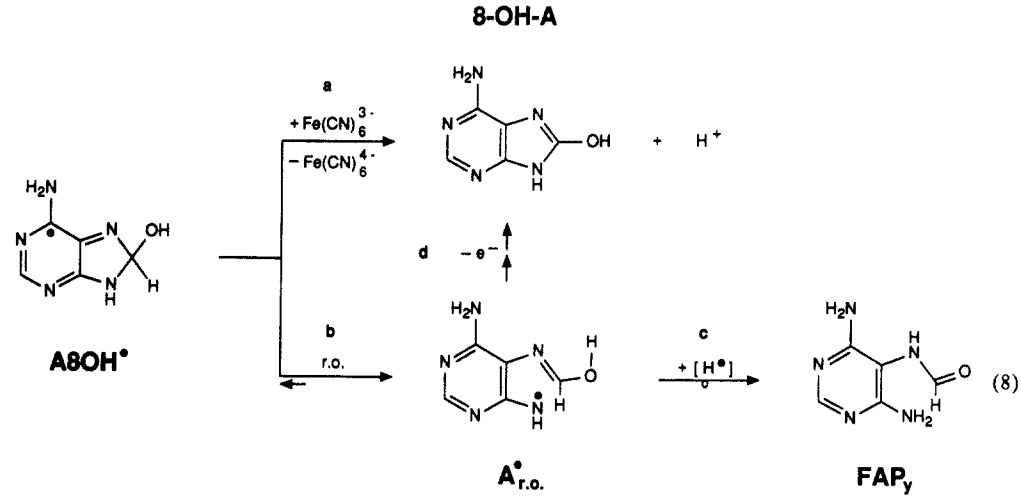
The effect of $Fe(CN)_6^{3-}$ on the yield of 8-OH-A is explained in terms of competition between (bimolecular) oxidation of A8OH• to give 8-OH-A (eq 8a, Scheme III) and (unimolecular) ring opening giving rise (eq 8b) to the ring-opened $A^•_{r.o.}$. Reduction of $A^•_{r.o.}$ gives FAPy (step 8c). Even in the absence of external oxidants the yield of FAPy is only 2% (Table II). This is understandable since the radical $A^•_{r.o.}$ exists in an oxidizing envi-

(36) Cordes, E. H. *Prog. Phys. Org. Chem.* **1967**, *4*, 1. Fife, T. H. *Acc. Chem. Res.* **1972**, *5*, 264. McClelland, R. A.; Santry, L. J. *Acc. Chem. Res.* **1983**, *16*, 394. Capon, B.; Dosunmu, M. I.; Sanchez, M. N. M. *Adv. Phys. Org. Chem.* **1985**, *21*, 37.

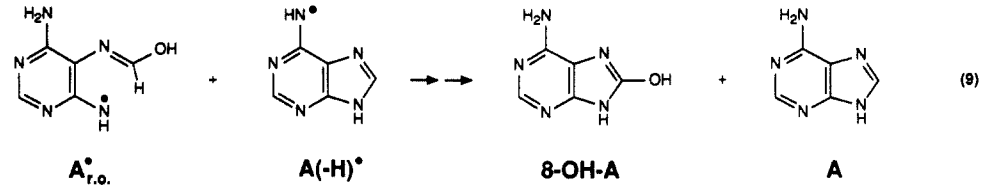
(37) Steenken, S.; Raghavan, N. V. *J. Phys. Chem.* **1979**, *83*, 3101. Raghavan, N. V.; Steenken, S. *J. Am. Chem. Soc.* **1980**, *102*, 3495, and references therein.

(38) Asmus, K.-D.; Deeble, D. J.; Garner, A.; Idriss Ali, K. M.; Scholes, G. *Br. J. Cancer* **1978**, *37* (Suppl. III), 46.

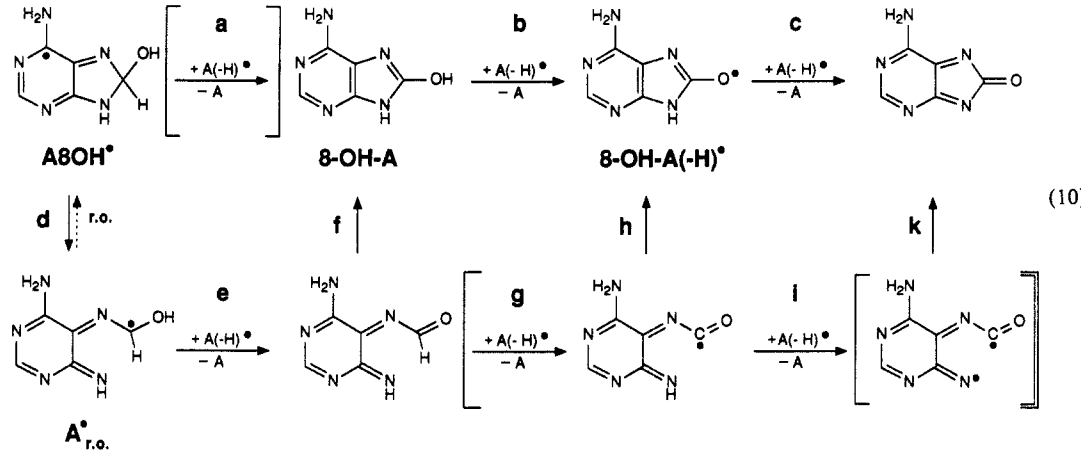
Scheme III



Scheme IV



Scheme V



ronment due to the excess of A(−H)• [81% of the OH radicals lead to the oxidizing A(−H)•; see section b1]. Under these conditions, the nonradical product FAPy can be formed only by disproportionation of $A^{\bullet}_{r.o.}$, a process that is not necessarily the only one by which this radical disappears.

From Figure 6 and Table II it is seen that even in the absence of added oxidant the yield of 8-OH-A is 5%, i.e., 3 times greater than that of FAPy. This implies that *oxidation* is the main way by which A8OH• disappears. As for the oxidant of A8OH•, the only reasonable candidate is A(−H)•.[39] However, under γ-radiolysis conditions the stationary concentration of A(−H)• is $\sim 10^{-9}$ M, so the reaction period for radical–radical interactions is of the order of seconds, a time much too long for A8OH• to survive intact, i.e., ring closed ($k_{\text{ring opening}} = 1.3 \times 10^5\ s^{-1}$, Table I). On this basis, the redox partner of A(−H)• *cannot* be A8OH•. There are two alternatives to explain the fact that 8-OH-A is at all formed. One is the assumption that the ring opening (eq 8b) is *reversible*. The other is that $A^{\bullet}_{r.o.}$ is oxidized by A(−H)• and *then* undergoes ring *closure* of the imidazole ring (see eq 8d and eq 9, Schemes III and IV).

The involvement of A(−H)• in the production of 8-OH-A was shown by a scavenging experiment. As demonstrated by O'Neill,[9a,17] A(−H)• is reduced by ascorbate (probably leading to restoration of adenine). When 0.1 mM ascorbate was added to a 1 mM adenine solution (pH 7) reacting with •OH, the yield of 8-OH-A dropped to 20% of the value in the absence of ascorbate (see Table II), which supports the involvement of A(−H)• in the production of 8-OH-A.

As seen from Figure 6, the yield of 8-OH-A (in the presence of 0.8 mM $Fe(CN)_6^{3-}$) decreases with increasing [H^+], starting at pH ~6, and also with increasing [OH^-] above pH 10. The decrease in the yield of 8-OH-A is in support of the H^+- and OH^--catalyzed ring opening of A8OH•, observed by pulse radiolysis for the case of adenosine and deoxyadenosine (see Figure 5).

It was also investigated whether there occurs any •OH addition at C2. If the corresponding OH adduct is one-electron oxidized by an oxidant such as 1 mM $Fe(CN)_6^{3-}$ (or the stronger $IrCl_6^{2-}$, 0.2 mM) followed by deprotonation from C2,[41] the compound

(39) A(−H)• is indeed a very strong oxidant. The lower limit for its reduction potential at pH ~7 can be estimated to be ≥1.6 V/NHE (using data from ref 40), which may be compared with 0.36 V for $Fe(CN)_6^{3-}$ or 0.87 V for the strong oxidant $IrCl_6^{2-}$.

(40) Jovanovic, S. V.; Simic, M. G. *J. Phys. Chem.* **1986**, *90*, 974. Candeias, L. P.; Steenken, S. *J. Am. Chem. Soc.* **1989**, *111*, 1094. Jovanovic, S. V.; Steenken, S.; Simic, M. G. *J. Phys. Chem.* **1990**, *94*, 3583.

2-hydroxy-6-aminopurine (isoguanine) is expected, which is easily identified by HPLC with electrochemical detection.[42] However, thorough analysis of γ-irradiated N_2O-saturated solutions of adenine revealed no isoguanine. Taking into account the radiation dose and the detection limit (0.1 μM isoguanine), it is calculated that not more than 2% of the •OH radicals add at C2. Such a low percentage is not unreasonable on the basis of the low electron density at C2[43] and the electrophilic character of •OH.

It has previously been noted that the yield of radiation-chemical degradation of purines is far less than expected on the basis of the known yields of the primary radicals from the radiolysis of water.[38,44] The data collected in Table II are an additional example for the resistance of adenine toward •OH radical induced decomposition. The low number for loss of adenine in the absence of oxidants (30%) obviously means that the remaining 70% of the initially formed adenine-derived radicals are "repaired", i.e., converted back to adenine. A simple way that this may happen is by reduction of the oxidizing A(−H)•. This requires the presence of a reducing radical, and $A^{\bullet}_{r.o.}$ and A8OH• are obvious candidates.

However, on the basis of the pulse radiolysis results, the yield of the oxidizing A(−H)• is 81%, so there remain only 19% for reducing radicals. This number is essentially the same as that (18%) for the yield of A8OH• [determined via 8-OH-A in the presence of $Fe(CN)_6^{3-}$]. Obviously, the 18% is far too low to explain the large yields (70%) of repair of adenine [determined in the absence of $Fe(CN)_6^{3-}$]. Therefore, additional reducing equivalents are needed. One possibility is 8-OH-A. In fact, on the basis of pulse radiolysis studies with $Br_2^{\bullet-}$ and Tl^{2+} as oxidants, it is evident that 8-OH-A is much more easily oxidized than adenine.[45] If it is assumed that A(−H)• is reduced by 8-OH-A (eq 10b, Scheme V), a *second* reducing equivalent is available, and further oxidation of the resulting radical, 8-OH-A(−H)•, would provide a third reducing equivalent, cf. eq 10c. If this is true it means that *one* A8OH• and its oxidation products are able to repair *three* A(H)• radicals. The ring-opened radicals could provide reducing equivalents in an analogous way (eq 10e–i). This admittedly speculative mechanism implies that the yield of depletion of adenine cannot be smaller that the yield of *reducing* radicals, and the experimental results (~30% depletion, ~20% reducing radicals) are not in disagreement with this picture. A further piece of evidence in favor of this is the enhancement of the yield of depletion of adenine by $Fe(III)(CN)_6^{3-}$ (Table II and ref 38). This oxidant removes reducing equivalents required to repair A(−H)•. The fact that even in the presence of the oxidant the depletion yield is not 100% can be accounted for by repair of A(−H)• by the $Fe^{II}(CN)_6^{4-}$ produced on reduction of $Fe^{III}(CN)_6^{3-}$. This reaction is strongly exothermic [E(A(−H)•) ≥ 1.6 V,[39] $E(Fe(CN)_6^{3-}$ = 0.36 V/NHE].

Summary and Conclusions

It has been demonstrated that on •OH reaction with adenine and its nucleosides and nucleotides two different types of radical are produced that undergo unimolecular transformation reactions for which the activation parameters and pH dependencies (for the nucleosides) have been determined. The more abundant of the isomeric radicals formed is identified as A4OH•, produced by addition of •OH at C4. This weakly oxidizing radical loses a molecule of water to give A(−H)•, which is a considerably stronger oxidant[17] due to more unpaired spin density at the exo- and endocyclic N's. The dehydration is inhibited by H^+. The other type of OH adduct is A8OH• which, in the absence of oxidants, undergoes ring opening, a reaction that is accelerated by H^+ as well as by OH^-.

The relatively low yield of decomposition of adenine by •OH is tentatively accounted for by repair of A(−H)• by reducing equivalents derived from A8OH•. However, a full understanding of the •OH-induced radical chemistry of adenine is still missing, and in order to achieve this, a thorough product analysis study leading to a complete material balance will have to be performed.

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(41) This mechanism is analogous to the conversion of A8OH• to yield 8-OH-A (see eq 8a).

(42) The solutions were analyzed for isoguanine immediately after irradiation to minimize any metal ion catalyzed decomposition, which however was sufficiently slow as shown by blank experiments with authentic isoguanine.

(43) Pullman, A.; Pullman, B. *Bull. Soc. Chim. Fr.* **1958**, 766. Pullman, B.; Pullman, A. *Quantum Biochemistry*; Interscience: New York, 1963; p 224.

(44) Scholes, G. in ref 3a, p 153.

(45) Candeias, L. P.; Steenken, S., unpublished results. An attempt was made to observe directly by pulse radiolysis the oxidation of 8-OH-A by A(−H)•. However, no reaction was seen at pH 7.4. The conclusion is that k(A(−H)• + 8-OH-A → A + 8-OH-A(−H)•) ≤ 1×10^7 M^{-1} s^{-1}.