Rate constants of ozone reactions with DNA, its constituents and related compounds

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The rate constants of the reaction of ozone with DNA, its constituents and related compounds have been determined as a function of pH by competition with nitrite and/or buten-3-ol and, when the rate constant was $\leq 10^3$ dm^3 mol^{-1} s^{-1}, by the indigo method. Depending on the degree of protonation, the rate constant (in units of dm^3 mol^{-1} s^{-1}) varies substantially, *e.g.* in the case of cytosine, $k = 18$ (protonated), $k = 1.4 \times 10^3$ (neutral) and $k = 1.5 \times 10^6$ (deprotonated). A similar variation has been found with the other nucleobases. Upon deprotonation the mechanism of the ozone reaction may also change; *e.g.* no singlet dioxygen (O_2 $^1\Delta_g$) is formed in its reaction with 5-chlorouracil, but when the 5-chlorouracilate ion predominates it becomes a major product (~42%). Rate constants for the neutral compounds are: thymine (4.2×10^4), thymidine (3.0×10^4), 1,3-dimethyluracil (2.8×10^3), uracil (650), 6-methyluracil (140), 5-chlorouracil (4.3×10^3), orotic acid (5.9×10^3), isoorotic acid (3.7×10^3), 2′-deoxycytidine (3.5×10^3), cytidine (3.5×10^3), adenine (12), 2′-deoxyadenosine (14), adenosine (16), guanosine (1.6×10^4), 2′-deoxyguanosine (1.9×10^4) and DNA (410). In the case of adenine and its derivatives, and thus also in the case of DNA, $^{\bullet}$OH is produced (*via* $O_2^{\bullet -}$ as an intermediate). For the determination of their intrinsic ozone rate constants, *tert*-butyl alcohol was added as the $^{\bullet}$OH scavenger.

In drinking-water processing, ozone is gaining in importance, because it is not only a good oxidant but also a powerful disinfectant [1] and readily copes with bacteria and viruses.[2–18] Mechanistic details of their inactivation are as yet not fully understood. Some 10^8 ozone molecules are required for the inactivation of a bacterium.[2,11] It has been suggested that this is due to a destruction of the bacterial cell wall and subsequent leakage of cellular contents.[2] However, ozone also causes mutations.[19–21] This may be taken as evidence for damage of its DNA (with the cell remaining adequately intact), but it cannot be excluded that ozone by-products (*e.g.* formed in the reaction with the cell wall) may have caused the mutagenic effect. For example, hydroperoxides and H_2O_2 are typical ozonation by-products, and the latter is known to be weakly mutagenic.[22,23] In contrast, the inactivation of viruses is considered to be mainly caused by ozone-induced damage of their nucleic acids, although damage of the proteins that make up the capsid also occurs.[7] The considerable spread of inactivation half-lives among various enteroviruses [10] may indicate that, depending on the structure of the virus, there is varying competition between ozone damage of the nucleic acid (lethal) and the proteins (less lethal).

There is already quite a body of information on the reaction of ozone with nucleic acids and their constituents.[24–35] However, in order to reach a better understanding of the inactivation of viruses and bacteria by ozone, a firm set of rate constants is required on which further studies can be based. The determination of the rate of reaction of ozone with the nucleic acids and their constituents *e.g.* by the stopped-flow technique is difficult because of the strong overlap of the absorption spectra of the nucleobases with that of ozone (for the data presently available see Table 1).

This has led us to use another approach: the application of competition kinetics.[36] Deprotonation or protonation of substrates has a dramatic effect on the rate of ozone reactions. The most striking example is phenol, whose ozone rate constant increases six orders of magnitude upon deprotonation.[37] As a consequence of this, the reaction of ozone with phenol is largely a reaction of ozone with the phenolate in equilibrium [pK_a(phenol) = 10], even in slightly acidic solution. Similarly, amines no longer react with ozone when protonated.[36,38] Also in the reaction of ozone with the nucleobases, one has to take their protonation–deprotonation equilibria into account. Thus, a detailed study covering a large pH range is required for a better understanding of the kinetics of the reaction of ozone with the nucleobases and with DNA. Such data, together with some additional information, will be presented in this paper.

Experimental

Thymine, 1,3-dimethyluracil, adenine (Fluka), uracil, cytosine, adenosine, 2′-deoxyguanosine, calf thymus DNA, buten-3-ol (Merck), 6-methyluracil (Janssen), 5-chlorouracil, orotic acid, isoorotic acid, cytidine (Sigma), thymidine, 2′-deoxycytidine, 2′-deoxyadenosine (Acros), guanosine (Boehringer Mannheim) and indigo trisulfonate (Riedel-de Haën) were used as received. Solutions were made up in Milli-Q-filtered (Millipore) water. Ozone was generated with the help of a dioxygen-fed ozonator (Philaqua Philoz 04, Gladbeck). The ozone content in the stock solutions was determined spectrophotometrically using ε(260 nm) [39,40] = 3300 dm^3 mol^{-1} cm^{-1}. DNA solutions were made up in phosphate buffer (pH 6.8, 1×10^{-3} mol dm^{-3}) by gently stirring overnight.

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In the present study, many rate constants have been determined by competition. Therefore, the principle of competition kinetics is briefly recalled. Two substrates (here, the competitor C and, *e.g.*, a nucleobase N) react with ozone [reactions (1) and

$$C + O_3 \longrightarrow P_1 \quad (1)$$

(2)]. While the product of the reaction of ozone with the com-

$$N + O_3 \longrightarrow P_2 \quad (2)$$

petitor (P_1) can be monitored, the products of the reaction of ozone with the nucleobase (P_2) remain silent.

At a given initial ozone concentration ($[O_3] \leqslant [C]$ and [N]), relationship (3) holds ($[P_1]_o$ is the concentration of the meas-

$$\frac{[P_1]}{[P_1]_o} = \frac{k_1[C]}{k_1[C] + k_2[N]} \quad (3)$$

ured product in the absence and $[P_1]$ in the presence of N), which can be reorganized to yield eqn. (4).

$$\frac{[P_1]_o}{[P_1]} = \frac{k_1[C] + k_2[N]}{k_1[C]} = 1 + \frac{k_2[N]}{k_1[C]} \quad (4)$$

Plotting $([P_1]_o/[P_1]) - 1$ *vs.* [N]/[C] yields a straight line with the slope of k_2/k_1. Since the rate constant of the competitor with ozone, k_1, is known, the rate constant of ozone with N, k_2, can be calculated.

As competitors, buten-3-ol and nitrite were used which yield 1 mol of formaldehyde [41] and nitrate,[42] respectively, per mole of ozone. Solutions containing substrate and competitor at varying ratios were mixed with an ozone solution, and the competitor-derived product was quantified. Formaldehyde was determined by the Hantzsch method [43] and nitrate by ion chromatography (Dionex DX-100, AS-14 column with AG-14 precolumn, eluent: 4.5×10^{-4} mol dm^{-3} Na_2CO_3–4.3×10^{-4} mol dm^{-3} $NaHCO_3$). When the rate constant to be determined is $\leq 10^3$ dm^3 mol^{-1} s^{-1}, competition with buten-3-ol is no longer feasible. In this range, the indigo method [44,45] was used. An ozone solution was added to a solution of the substrate present in ≥10-fold excess over the ozone concentration. The remaining ozone was titrated as a function of reaction time by the addition of indigo trisulfonate,[44,45] which is practically instantaneously ($k = 9.4 \times 10^7$ dm^3 mol^{-1} s^{-1}) [36] bleached by the ozone still present.

Around neutrality, the stock solutions were buffered with phosphate to prevent a drop in pH due to the formation of acids that are released in the course of the reactions of ozone at least with the pyrimidine nucleobases.[35]

The pK_a value of 6-methyluracil was redetermined by measuring the changes in the UV absorption spectra as a function of pH (Perkin-Elmer, Lambda 16).

Stopped-flow experiments were carried using a Biologic SFM3 set-up.

Results and discussion

Around pH 7, all nucleobases are present largely in their uncharged forms (*cf.* Table 1). In the case of the uracil family and guanine derivatives, protonation occurs only at very low pH. On the other hand, cytosine, adenine and their derivatives become protonated already at around pH 4. At high pH, the uracil family are deprotonated at pH ~ 9.8, as long as one of the nitrogens remains unsubstituted. In thymine, for example, the second nitrogen may also be deprotonated at very high pH. Most of the reported pK_a values of 6-methyluracil centre around 9.8 as well,[46] but because of the importance of this

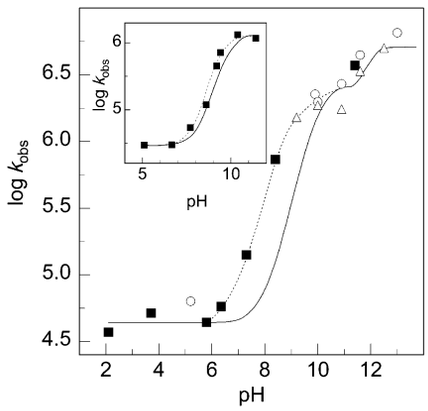


Fig. 1 Observed rate constant for the reaction of thymine with ozone as a function of pH. The solid line is calculated on the basis of the rate constants measured at pH 2, 11 and 13 and the established pK_a values of 9.8 and 12.5. Inset: analogous data for thymidine. Competition with buten-3-ol (■) and nitrite in the absence (○) and presence (△) of *tert*-butyl alcohol.

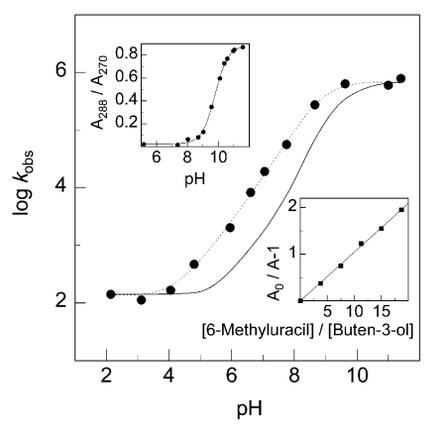


Fig. 2 Observed rate constant for the reaction of 6-methyluracil with ozone as a function of pH. The solid line is calculated on the basis of the rate constants measured at pH 2 and 11 and the pK_a value of 9.8 based on the data in the upper inset. Lower inset: a typical competition plot using buten-3-ol as competitor.

value for our study, we have remeasured it by following the change of the ratio of the absorptions at 288 and 270 nm as a function of pH (*cf.* inset in Fig. 2), and our value of 9.8 is in agreement with the majority of the literature data.

As can be seen from Table 1, the rate constants span more than five orders of magnitude. When the rate constant is small ($<10^3$ dm^3 mol^{-1} s^{-1}), the indigo method is most suited. For higher rate constants buten-3-ol ($k = 7.9 \times 10^4$ dm^3 mol^{-1} s^{-1})[41] is usually used as competitor. At pH > 10, the competition with buten-3-ol no longer yields fully reliable values. For unknown reasons, the formaldehyde yields tend to be less reproducible; they are typically lower than expected. In addition, when *tert*-butyl alcohol is added as an $^{\bullet}OH$ scavenger, buten-3-ol can no longer be used as the competitor, since *tert*-butyl alcohol also yields formaldehyde in $^{\bullet}OH$-induced reactions (~30%).[47] Thus, under these conditions we used nitrite as the competitor for which rate constants of 3.7×10^5, 3.3×10^5 (at room temperature) and 1.6×10^5 dm^3 mol^{-1} s^{-1} (at 11 °C) are reported.[48] Our experiments were done at 21(±1) °C, and at this temperature the rate constants of the reaction of ozone with buten-3-ol and nitrite were redetermined using the stopped-flow technique under the condition of first-order kinetics (ten-fold excess of substrate) to be 9.1×10^4 (*cf.* ref.49) and $(3.5–4.0) \times 10^5$ dm^3 mol^{-1} s^{-1}, respectively, *i.e.* in agreement with the literature values. To ensure internal consistency, the competition of buten-3-ol *vs.* NO_2^- was studied. This can be carried out by measuring either formaldehyde or nitrate yields [*cf.* reactions (5) and (6)], *e.g.* as a function of the [buten-3-ol]/[NO_2^-] ratio.

$$CH_2{=}CHCH(OH)CH_3 + O_3 \longrightarrow CH_2O + CH_3CH(OH)C(O)H + H_2O_2 \quad (5)$$

$$NO_2^- + O_3 \longrightarrow NO_3^- + O_3\,(O_2{}^1\Delta_g) \quad (6)$$

The formaldehyde assay [43] is sensitive to elevated NO_2^- concentrations which suppress the colour formation, and therefore the competition had to be done at low concentrations of the competing substrates. Nitrate determination by ion chromatography is not affected by the presence of the reactants. These two data sets yield $k(NO_2^-)/k$(buten-3-ol) ratios of 5.4 and 5.7. Taking the literature data ($k(NO_2^-) = 3.7 \times 10^5$ and k(buten-3-ol) = 7.9×10^4 dm^3 mol^{-1} s^{-1} mentioned above) one arrives at a ratio of 4.7. Considering the inherent uncertainties in all of these data, this is an acceptable agreement.

In their reactions with ozone, nitrogen-containing compounds, *e.g.* amines,[38] can give rise to the formation of $^{\bullet}$OH, and the ensuing $^{\bullet}$OH-induced reactions can distort the kinetics considerably. For this reason, the $^{\bullet}$OH scavenger *tert*-butyl alcohol was added to test whether its presence has an influence on the rate of reaction.[50] As will be shown below, this precaution is only necessary in the case of adenine and its derivatives, *i.e.* also in DNA in which case it is essential.

The rate constants determined in the present study are compiled in Table 1.

Uracil, thymine and their derivatives

The pH dependencies of the observed bimolecular rate constants of ozone with thymine and 6-methyluracil are shown in Figs. 1 and 2. Under conditions where their neutral forms predominate, the reaction is relatively slow, but with increasing pH the rate of reaction increases. The position of the methyl group at the C(5)–C(6) double bond has a noticeable effect on the rate of reaction. Comparison of the values at low pH shows that thymine reacts more than two orders of magnitude faster than 6-methyluracil. The other uracil derivatives fall in between. In contrast, the rate constants of the corresponding deprotonated forms are very similar: they all center around 10^6 dm^3 mol^{-1} s^{-1} (*cf*. Table 1).

Upon raising the pH, the rate of reaction increases (Figs. 1 and 2), and in the case of thymine, experiments were extended into the pH range where the second nitrogen is deprotonated, and a further increase in rate is observed (Fig. 1). At pH 13, a value of 6.5×10^6 dm^3 mol^{-1} s^{-1} is found and attributed to the rate constant for doubly deprotonated thymine. The determination of rate constant for the mono-deprotonated thymine is fraught with some error and can only be estimated from the inflection point near pH 10, from which we obtain a value of ~3×10^6 dm^3 mol^{-1} s^{-1}.

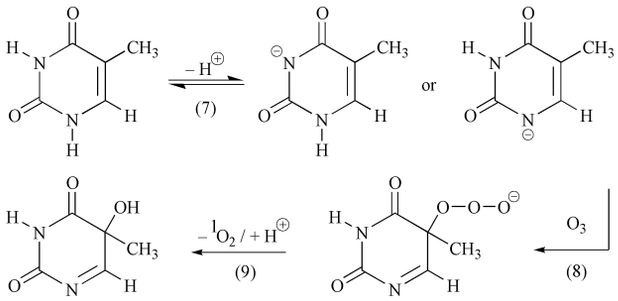
As can be seen from Figs. 1 and 2, there is a considerable disagreement between the measured pH dependencies (dotted lines) and the ones calculated on the basis of the pK_a values and rate constants given in Table 1 (solid lines). The experimental curves suggest reactions of compounds that are noticeably more acidic than their pK_a values would indicate. In the reaction of ozone with olefins, there is evidence at low temperatures for the formation of a charge transfer complex which subsequently decays into products.[54,55] If in the present case such a charge transfer complex is sufficiently long-lived it could turn more acidic than its parent. As a consequence of this, the rate of reaction could be higher than the one calculated on the basis of the pK_a value of the parent. We are aware that spontaneous deprotonation of these compounds is slow due to their high pK_a values (~0.1–1 s^{-1}), but the presence of buffer, which had to be added to maintain the pH, speeds up the rate of deprotonation. Nevertheless, the ozone charge transfer complex would have to be more than one unit more acidic and also quite long-lived to show this effect. In this context, it is intriguing that only a small, if any, deviation is found for thymidine (inset in Fig. 1), uracil and 5-chlorouracil (data not shown) and also no such deviation is found for the cytosine, adenine and guanine systems (see below).

The formation of $^{\bullet}$OH as the reason for this unexpected deviation has been excluded for the thymine and 6-methyluracil systems. This has been done by adding *tert*-butyl alcohol in excess (in the absence of a competitor) at around the pH where the deviations are most noticeable. No formaldehyde (due to $^{\bullet}$OH-induced *tert*-butyl alcohol degradation) was detected. This is in agreement with the observation that at high pH addition of *tert*-butyl alcohol had no effect on the rate of reaction (nitrite as competitor).

It has been mentioned above that there is an increase in rate in the region where the deprotonation of the second nitrogen of thymine occurs. The rate constant of this doubly deprotonated species might have been underestimated (experiments could not be extended beyond pH 13; the high salt load prevented nitrate determinations by ion chromatography). Yet the marked deviation of the data in the pH range 9–12.5 from a slope of unity indicates that even the assumption of a much higher rate constant for doubly charged thymine cannot explain the observed deviation from the expected pH dependence.

It is remarkable that the position of the methyl group, *i.e.* when the rate of reaction with thymine is compared with that with 6-methyluracil, has such a dramatic effect (a factor of 300, *cf*. Table 1). Considerable differences in the rate constants of isomers, *e.g.* 1,1-dichloroethene (k = 110 dm^3 mol^{-1} s^{-1}), (*Z*)-1,2-dichloroethene (k = 540 dm^3 mol^{-1} s^{-1}) and (*E*)-1,2-dichloroethene (6500 dm^3 mol^{-1} s^{-1}) have been noticed before,[41] but in these cases the rate constants differed at most by a factor of 60. Even more surprising is the observation that the rate constant for 6-methyluracil is *lower* than that for uracil. Typically, an additional methyl group at the reacting C=C double bond *increases* the rate of reaction by a factor of ~4.[41] A very marked difference between these uracil derivatives and simple olefins is also noticed when 6-methyluracil is compared with 5-chlorouracil. One would have expected that the former reacts considerably faster with ozone than the latter, but the reverse has been observed (*cf*. Table 1).

The observed increase in rate upon deprotonation of thymine is connected with a change in the reaction mechanism. While the neutral nucleobase does not give rise to the formation of singlet dioxygen (O_2 $^1\Delta_g$), deprotonated thymine yields O_2 $^1\Delta_g$ in ~8% yield.[42] Possibly, it is formed *via* reactions (7)–(9) in Scheme 1. Thymine is deprotonated at N(1) as well as at N(3)



Scheme 1

[equilibrium (7)]. Dipolar addition of ozone to thymine deprotonated at N(1) gives rise to an isopyrimidine hydrotrioxide [reaction (8)]. Since the chemistry of isopyrimidines is known,[56,57] a product study, which is under way, may help to elucidate mechanistic details.

Thus far, our mechanistic proposal is supported by the observation that thymidine, which can be deprotonated only at N(3), does not afford any singlet dioxygen at high pH.[42] In this context, it may be worth mentioning that 5-chlorouracil shows this change in mechanism even more strongly: no singlet dioxygen (O_2 $^1\Delta_g$) is formed at pH ~3.5, but when the 5-

Table 1 Compilation of the rate constants for the reaction of ozone with nucleobases and related compounds (in units of $dm^3\ mol^{-1}\ s^{-1}$) at different stages of protonation. Literature values [27] are in parentheses

Substrate	pK_a values[a]	Protonated	Neutral	Deprotonated
Thymine	9.9, >12	—	4.2×10^4 (2.3×10^4)	$\sim 3 \times 10^6$
Thymidine	9.8	—	3.0×10^4	1.2×10^6
5′-dTMP	10.0	—	(1.6×10^4)	
1,3-Dimethyluracil	—	—	2.8×10^3	—
Uracil	9.5, >13	—	650	9.2×10^5
6-Methyluracil	9.8	—	140	6.0×10^5
5-Chlorouracil	8.0	—	4.3×10^3	1.3×10^6
Orotic acid[b]	2.1, 9.45	—	5.9×10^3	nd[c]
Isoorotic acid[b]	4.2, 8.9	—	3.7×10^3	nd
Cytosine	4.6, 12.2	18	1.4×10^3 (930)	1.5×10^6
2′-Deoxycytidine	4.3	44	3.5×10^3	—
Cytidine	4.15	40	3.5×10^3	—
5′-dCMP	4.6		(1.4×10^3)	
Adenine[d]	4.15, 9.8	5	12	1.3×10^5
2′-Deoxyadenosine[d]	3.8	5	14	[e]
Adenosine[d]	3.5	5	16	[e]
5′-dAMP	4.4		(200)	
Guanosine	2.5, 9.2	<300	1.6×10^4	4.0×10^6
2′-Deoxyguanosine	2.5, 9.2	nd	1.9×10^4	nd
5′-dGMP	2.9, 9.7		(5×10^4)	
DNA		—	410[d,f]	—

[a] pK_a values taken from ref. 51, 5-chlorouracil,[52] orotic acid and isoorotic acid.[53b] [b] Rate constant determined at pH 7, at which the carboxylate group is deprotonated, but not one of the nitrogens. [c] nd = not determined. [d] In the presence of 0.2 mol dm^{-3} *tert*-butyl alcohol. [e] See text. [f] The average molecular weight of the nucleotides in DNA is taken as 350 Da.

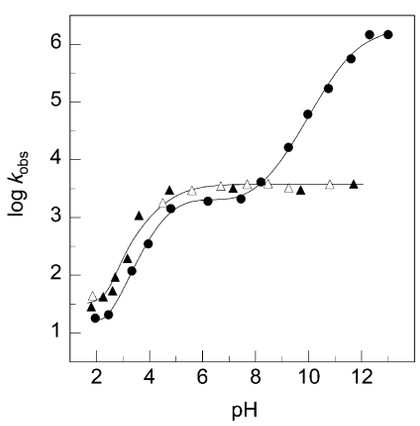


Fig. 3 Observed rate constant for the reaction of ozone with cytosine (●), cytidine (▲) and 2′-deoxycytidine (△) as a function of pH. The solid line is calculated on the basis of the rate constants and the pK_a values given in Table 1.

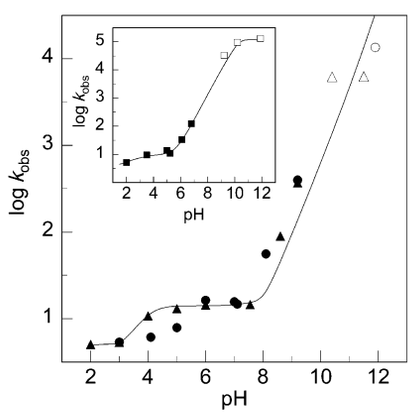


Fig. 4 Observed rate constant for the reaction of ozone with adenosine (●, ○), and 2′-deoxyadenosine (▲, △) as a function of pH; the solid line is calculated on the basis of an assumed limiting rate constant of $1.3 \times 10^5\ dm^3\ mol^{-1}\ s^{-1}$, as in adenine, and the pK_a values given in Table 1. Inset: adenine; the solid line is calculated on the basis of the rate constants and pK_a values given in Table 1. Solid symbols refer to experiments using the indigo method, open symbols to competition kinetics with nitrite as competitor. In all experiments *tert*-butyl alcohol (0.1 mol dm^{-3}) was added as an $^{\bullet}OH$ scavenger.

chlorouracilate ion predominates it becomes a major product (~42%).[42]

Cytosine, cytidine and 2′-deoxycytidine

As can be seen from Fig. 3, cytidine and 2′-deoxycytidine react somewhat faster than the free base. This is most likely due to the electron-donating property of the sugar moiety. Upon protonation their rates of reaction drop by two orders of magnitude. Whereas the nucleosides do not show an increase in rate at high pH, cytosine does. At high pH, the latter deprotonates (pK_a = 12.2). A similar pK_a value (pK_a = 12.5) is reported for cytidine, but here deprotonation is suggested to occur at C(2′).[58] Apparently deprotonation of the sugar moiety has practically no effect on the reactivity of these compounds with ozone.

Adenine, adenosine and 2′-deoxyadenosine

In the case of adenine and its derivatives, the addition of *tert*-butyl alcohol on the observed rate of reaction (Fig. 4) has a dramatic effect (*cf.* also refs.27,32). In its absence, the rate constant with adenine became too fast for the indigo method, *i.e.* the apparent bimolecular rate constant must have been faster than $\sim 10^3\ dm^3\ mol^{-1}\ s^{-1}$. This points to the intermediacy of $^{\bullet}OH$ at one stage. Indeed, when *tert*-butyl alcohol was added in large excess (0.1 mol dm^{-3}), we observed the formation of considerable amounts of formaldehyde (~0.2 mol per mol ozone) in the case of adenine as well as 2′-deoxyadenosine. Formaldehyde is an important product of the $^{\bullet}OH$-induced oxidation of *tert*-butyl alcohol.[47] The observed formaldehyde yield of ~20% relates to ~60% $^{\bullet}OH$ (the use of *tert*-butyl alcohol in the determination of $^{\bullet}OH$ yield in ozonation reactions will be published elsewhere) [50]. The reduction potential of adenine and its derivatives [59] is too high for an electron transfer to occur in the ozone reaction (note that in the case of guanosine, which has a much lower reduction potential,[59,60] no $^{\bullet}OH$ is formed, see below). Thus there must be another reason for the intermediacy of $^{\bullet}OH$ in this system. It will be shown elsewhere in more detail that the precursor of $^{\bullet}OH$ is most likely $O_2^{\bullet -}$. Here, it suffices to mention that tetranitromethane added as an $O_2^{\bullet -}$ scavenger is substantially degraded (*via* the intermediacy of trinitromethane anions). Addition of *tert*-butyl alcohol reduces the observed rate by a factor of more than two. This indicates that the

radicals that are formed upon $^{\bullet}OH$ attack on adenine/2′-deoxyadenosine must induce a chain reaction. The reaction of ozone with the cyanide ion also proceeds *via* a chain reaction,[61,62] which the *tert*-butyl alcohol cannot fully suppress, *i.e.* there must be an additional chain carrier. A similar situation may prevail here, but further work will be required to elucidate this system in more detail.

In the case of adenine, the pH dependence of the rate constant can be adequately fitted by the rate constants and the pK_a values given in Table 1 (*cf.* solid line in the inset of Fig. 4). The rate of reaction with adenosine and 2′-deoxyadenosine also increases with increasing pH, and these data can be fitted (solid line in Fig. 4) if the pK_a of 12.5 and the limiting rate constant of adenine are taken. It has been noted above that in the case of the cytosine nucleosides no further increase of the rate of reaction has been observed in the high pH range (*cf.* Fig. 3). This has been explained by the fact that the pK_a value at very high pH is attributed to a pK_a value of the sugar moiety and that these anions do not react with a rate higher than 3.5×10^3 dm^3 mol^{-1} s^{-1} given by neutral cytidine/2′-deoxycytidine themselves. At the highest pH that can be measured with confidence, the adenosine/2′-deoxyadenosine rate constant approaches ~10^4 dm^3 mol^{-1} s^{-1}, not much higher than the above value. There is no indication that this value is a limiting one, and indeed it is impossible to fit the data to such a low value. The solid line in Fig. 4 was constructed by taking the pK_a of 12.5 and the limiting value of the adenine system (1.3×10^5 dm^3 mol^{-1} s^{-1}). This is only acceptable as long as the pK_a at ~12.5 is due to the deprotonation of the adenine moiety. We have remeasured the absorption spectrum of 2′-deoxyadenosine at pH 14, and at the long-wavelength band no changes, in either ε or λ_{max}, with respect to its spectrum at pH 7 are observed (below 230 nm, the high absorption of OH^- prevents definite measurements). This would be in agreement with the earlier conclusion [63,64] that the pK_a at ~12.5 is due to a deprotonation of the sugar moiety, but not with the observed high reactivity. Thus, the reason for this unexpected observation remains unresolved.

The earlier determination of the rate constant of an adenine derivative, 5′-dAMP,[27] deserves comment. Its rate constant was estimated at 2×10^2 dm^3 mol^{-1} s^{-1} (in the absence of *tert*-butyl alcohol) by measuring its degradation relative to that of dCMP for which a rate constant of 1.4×10^3 dm^3 mol^{-1} s^{-1} had been determined by the stopped-flow technique under close to second-order conditions. In the presence of *tert*-butyl alcohol, the relative rate constant dropped by nearly an order of magnitude. In the view of this, these data agree reasonably with our value of ~15 dm^3 mol^{-1} s^{-1}. It was mentioned above that in the absence of *tert*-butyl alcohol we estimate that the observed rate constant for adenine and its derivatives is ≥10^3 dm^3 mol^{-1} s^{-1}. This is higher than the value of 2×10^2 dm^3 mol^{-1} s^{-1} obtained in the dCMP competition. This is not surprising. When $^{\bullet}OH$ radicals play a significant role in this system, they also attack dCMP, which is subsequently consumed, *i.e.* the estimate of the dAMP rate constant comes out too low.

Guanosine and 2′-deoxyguanosine

Guanosine and 2′-deoxyguanosine react about equally fast with ozone (Table 1). Upon deprotonation, the rate of reaction increases 250-fold. As can be seen from Fig. 5, the experimental data can be adequately fitted using the rate constants and pK_a values given in Table 1 (experiments at very low pH indicate that upon protonation the rate constant falls below 300 dm^3 mol^{-1} s^{-1}, data not shown). Addition of *tert*-butyl alcohol had no effect on the rate of reaction nor was any formaldehyde formed under these conditions. This excludes any electron transfer from the guanine moiety to ozone, although guanine has the lowest reduction potential of all the nucleobases [59,60] and, especially in basic solutions where its anion predominates,

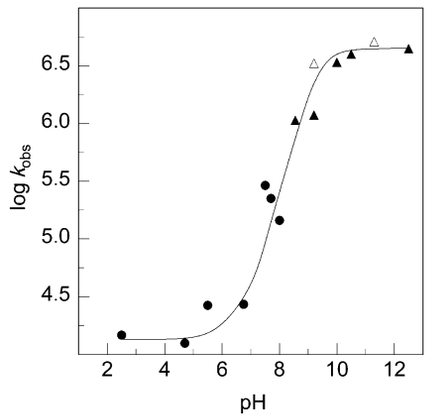


Fig. 5 Observed rate constant for the reaction of ozone with guanosine as a function of pH; the solid line is calculated on the basis of the rate constants and the pK_a value given in Table 1. Competition with buten-3-ol (●) and nitrite with (△) and without (▲) *tert*-butyl alcohol (0.1 mol dm^{-3}).

it is readily oxidized by many otherwise only weakly oxidizing agents (*cf.* ref.65).

DNA

As has been noticed before,[32] $^{\bullet}OH$ plays an important role in the reaction of ozone with DNA. From ref.27 and the present study, it is clear that $^{\bullet}OH$ radical formation must be due to the reaction of ozone with the adenine moiety. For the determination of the intrinsic ozone rate constant with DNA, *tert*-butyl alcohol has to be added. Under such conditions, the rate of reaction of DNA is only 410 dm^3 mol^{-1} s^{-1} (in the absence of *tert*-butyl alcohol $k_{obs} = 1.1 \times 10^3$ dm^3 mol^{-1} s^{-1}), *i.e.* much lower than that of the weighted average of the nucleobases. In the case of $^{\bullet}OH$, which reacts with the nucleobases and their derivatives at close to diffusion-controlled rates ($k \sim 3 \times 10^9$ dm^3 mol^{-1} s^{-1}),[66] the rate of reaction of $^{\bullet}OH$ with DNA is considerably lower ($k = 2.5 \times 10^8$ dm^3 mol^{-1} s^{-1}),[67] since, in this non-homogeneous reaction with the macromolecule DNA, two terms, a diffusion term (k_{diff}) and a reaction term (k) have to be considered.[68] The observed overall rate constant (k_{obs}) is the harmonic mean of these two rate constants [*cf.* eqn. (10)].

$$\frac{1}{k_{obs}} = \frac{1}{k} + \frac{1}{k_{diff}} \qquad (10)$$

Since, in contrast to $^{\bullet}OH$, ozone reacts with the nucleobases at rates much below the diffusion-controlled limit, the second term must fall away, and the rate of reaction of ozone with the nucleic acids is only given by the first term, *i.e.* it should be close to that of the weighted average of the concentrations of the various nucleobases in the nucleic acid times their rate constants with ozone. This is not observed. Structural effects such as hydrogen bonding between the nucleobases may be a reason for the strong reduction in rate.

Corresponding experiments with RNA were not carried out, because it could not be guaranteed that the commercially available RNA would be of similar purity and sufficiently double-stranded to yield complementary data. From the rate constants given in Table 1, one would assume that in RNA the guanine moiety is the most likely one to become degraded upon ozone treatment. This has indeed been observed.[24] In DNA, the situation might be somewhat different: besides guanine, thymine may be the other preferred target.

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