# The reactions of thymine and thymidine with ozone

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The ozonolysis of thymine and thymidine has been investigated by a product study complemented by kinetic studies using spectrophotometry, conductometry and stopped-flow with optical and conductometric detection. Material balance has been obtained. Ozonolysis of thymine ($k = 3.4 \times 10^4$ dm$^3$ mol$^{-1}$ s$^{-1}$) leads to the formation of the acidic (p$K_a = 4$) hydroperoxide 1-hydroperoxymethylene-3-(2-oxopropanoyl)urea **5** (~34%), neutral hydroperoxides (possibly mainly 1-hydroperoxyhydroxymethyl-3-(2-oxopropanoyl)urea **6**, total ~41%) and $H_2O_2$ (25%, with corresponding formation of 1-formyl-5-hydroxy-5-methylhydantoin **11**). The organic hydroperoxides decay (~$1.1 \times 10^{-3}$ s$^{-1}$ at 20 °C, $1.3 \times 10^{-4}$ s$^{-1}$ at 3 °C) releasing formic acid (formation of 5-hydroperoxy-5-methylhydantoin **18**) and also to some extent $H_2O_2$ (and **11**). After 100 min, the formic acid yield is 75%. Upon treatment at high pH, it increases to 100%. Reduction of the organic hydroperoxides with bis(2-hydroxyethyl) sulfide ($k = 50$ dm$^3$ mol$^{-1}$ s$^{-1}$) leads to **11** whose subsequent treatment with base yields 5-hydroxy-5-methylhydantoin **13** in 100% yield. It is suggested that the Criegee ozonide formed upon reaction with ozone at the C(5)–C(6) double bond opens heterolytically in two directions with subsequent opening of the C(5)–C(6) bond. In the preferred route (75%), the positive charge resides at C(6). Deprotonation at N(1) gives rise to **5**, while its reaction with water yields **6**. Loss of formic acid yields 5-hydroperoxy-5-methylhydantoin **18**. Reduction of **5** and **6** with the sulfide yields **11**. In the minor route (25%), the positive charge remains at C(5) followed by a reaction with water. The resulting α-hydroxy hydroperoxide rapidly loses $H_2O_2$ (formation of **11**). In basic solution, singlet dioxygen is formed (8%). The concomitant product, 5,6-dihydroxy-5,6-dihydrothymine has been detected. In the ozonolysis of thymidine, the rapid formation of conductance ($k = 0.55$ s$^{-1}$) is due to the release of acetic acid (18%). In this reaction a short-lived hydroperoxide is destroyed. As a consequence of this, 25 s after ozonolysis the total hydroperoxide yield is only ~78% (including 8% $H_2O_2$). The products corresponding to acetic acid are suggested to be $CO_2$ and *N*-(2-deoxy-β-D-erythropentofuranosyl)formylurea **22**. A number of organic hydroperoxides have been detected by HPLC by post-column derivatisation with iodide. An acidic hydroperoxide such as **5** in the case of thymine is not among the products. Upon sulfide reduction, the organic hydroperoxides yield mainly (43–50%) $N_1$-(2-deoxy-β-D-erythropentofuranosyl)-5-hydroxy-5-methylhydantoin **23**. The reasons for some striking differences in the ozonolyses of thymine and thymidine are discussed.

## Introduction

In drinking-water processing, ozone is gaining in importance, because it is not only a good oxidant but also a powerful disinfectant which readily copes with bacteria and viruses.[1–4] Mechanistic details of their inactivation are as yet poorly understood.

In the inactivation of viruses by the much more reactive OH radical (for compilations of ozone and OH radical rate constants see refs. 5,6), two targets have been recognised, the protein coat and the DNA(RNA).[7] Apparently, a good fraction of the OH radicals can penetrate through the protein coat and react with the nucleic acids located inside. In contrast, in the case of bacteria, OH radicals created in the bulk water are effectively scavenged by the material that makes up the bacterial membrane, and the resulting damage is insufficient to kill the bacterium.[7] In the case of the inactivation of viruses by ozone, DNA damage may also be a major cause, since in proteins only a few of their constituents, cysteine, cystine, tryptophan and tyrosine, react fast with ozone.[5] Less can be predicted for the inactivation of bacteria by ozone.

Ozone has also been known for a long time to cause damage to DNA of eukariotic cells, *e.g.*, in yeast,[8] in plants[9] and also in human cells.[10] Although ozone toxicity is of considerable concern in industrial countries resulting in daily reports on ozone levels in air, very little information as to the mechanism of its DNA damaging action is as yet available.

The reaction of ozone with nucleic acids and their constituents has already attracted some attention.[11–21] Recently, we have supplemented the available data on ozone rate constants with the nucleobases (the sugar moiety does not react with ozone at a comparable rate),[22] and from this study it follows that the guanine and thymine moieties are considerably more reactive than the cytosine and adenine ones.

The reaction of ozone with thymidine has already been studied,[20] but a number of questions, especially as to mechanistic details, remained open. For this reason, we have reinvestigated the thymidine system based on what we have learned from a detailed study on the products, part of them short-lived intermediates, of its base moiety, thymine.

## Experimental

Thymine (Fluka) and thymidine (Acros) were used as received. The reagents bis(2-hydroxyethyl) sulfide (Aldrich) and catalase (Boehringer Mannheim) and the reference material 5-hydroxy-5-methylhydantoin (Toronto Research Chemicals) were also

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commercially available. Ozone was generated with the help of a dioxygen-fed ozonator (Philaqua Philoz 04, Gladbeck). The ozone content of the aqueous ozone stock solutions was determined spectrophotometrically using $\varepsilon(260\ nm)$[23,24] = 3300 $dm^3\ mol^{-1}\ cm^{-1}$. Solutions were made up in Milli-Q-filtered (Millipore) water. For ozonation, stock substrate-containing solutions were mixed with an aliquot of the ozone stock solution.

Formic and acetic acids were determined by ion chromatography (Dionex DX-100, AS-9 HC, eluent: $1 \times 10^{-2}$ mol $dm^{-3}$ $NaHCO_3$ at 1 ml $min^{-1}$).

Liquid chromatography–mass spectrometry (LCMS) measurements (Hewlett-Packard, HP1100/HP5989B) were carried out by electrospray ionisation (ESI) in the positive mode using acetonitrile–water (1 : 1) as eluent.

Hydrogen peroxide and organic hydroperoxides were determined with molybdate-activated iodide.[25] This reagent consists of two components. One contains iodide (0.4 mol $dm^{-3}$), molybdate ($1.6 \times 10^{-4}$ mol $dm^{-3}$) and KOH ($3.6 \times 10^{-2}$ mol $dm^{-3}$), the other potassium hydrogen phthalate (0.1 mol $dm^{-3}$) as buffer (pH ~4).[25] Equal volumes of the probe and the reagents are mixed, and the formation of $I_3^-$ is measured at 350 nm ($\varepsilon$ = 25500 $dm^3\ mol^{-1}\ cm^{-1}$). Some experiments have also been carried out without molybdate as catalyst to differentiate between highly reactive hydroperoxides and hydrogen peroxide. The latter reacts very slowly under these conditions.

For gas chromatography–mass spectrometry (GCMS), a Hewlett-Packard (5890 Series II) setup was used. For better volatility, the samples were trimethylsilylated (TMS) with bis(trimethylsilyl)trifluoroacetamide (BSTFA, Macherey und Nagel). 5-Hydroxy-5-methylhydantoin yields the 3-TMS derivative, MW = 346 Da, *m/z* (%): 331 (100), 216 (7), 147 (46), 73 (63). The *cis*/*trans* isomers of 5,6-dihydro-5,6-dihydroxythymine (thymine glycol) were identified as their 4-TMS derivatives, MW = 448 Da, *m/z* (%): 433 (10), 405 (8), 331 (75), 147 (25), 73 (100).

Stopped-flow experiments were carried out using a Biologic SFM3 and a laboratory-made set-up. This allowed us to follow the kinetics of the reaction either spectrophotometrically with a diode array detector (Tidas-16, J&M, Aalen) or conductometrically. For this purpose, a laboratory-made setup (similar to the one described before)[26,27] has been used.

Conductometric measurements on the longer time-scale were carried out with a conductometer (CDM3, Radiometer).

## Results and discussion

### The general reaction mechanism of ozone with olefins in aqueous solution

According to a detailed rate and product study that also involved the characterisation of short-lived hydroperoxides, there is a considerable influence of the electron-donating/withdrawing power of substituents (**D** *vs.* **A**) on the final products [*cf.* reactions (1)–(5)].[28]

In fact, an electron-donating group **D** such as a methyl substituent favours the formation of the α-hydroxy hydroperoxide at this position [reactions (2) and (4)]. This has been rationalised by a stabilisation of the carbocation formed in reaction (2).

In the reaction with thymine and thymidine, ozone will add to the C(5)–C(6) double bond. Due to the electron-donating properties of N(1), the Criegee intermediate formed in reaction (1) will preferentially open according to reaction (2). It will be shown that the substituents at N(1) (H in thymine, 2-deoxyribosyl in thymidine) have a pronounced effect on the subsequent reactions of the carbocation formed in reaction (2). In thymidine, this short-lived intermediate can only react according to reaction (4), but in thymine it can also deprotonate at N(1).

### Thymine

Thymine **1** reacts with ozone with a rate constant of $4.2 \times 10^4$ $dm^3\ mol^{-1}\ s^{-1}$, and upon deprotonation ($pK_a$ = 9.9) the rate constant increases to $3 \times 10^6$ $dm^3\ mol^{-1}\ s^{-1}$.[22] At pH 6.5, where the experiments to be reported next have been carried out, the contribution of the anion in product formation can be neglected. Under typical experimental conditions ([thymine] = $1 \times 10^{-3}$ mol $dm^{-3}$, $[O_3] = 1 \times 10^{-4}$ mol $dm^{-3}$) the ozone half-life is 0.016 s, *i.e.* the reaction proceeds at the time scale of the mixing of the two solutions.

**Conductometry and ion chromatography.** When the ozonolysis is carried out in a conductometric cell, the conductance rises with biphasic kinetics (Fig. 1). The first step is too fast to be resolved kinetically by means of conventional conductometry (see Fig. 1). The second shows a half-life of 10.5 min at room temperature ($k \approx 1.1 \times 10^{-3}\ s^{-1}$). At 3 °C, the conductance build-up is eight times slower ($k \approx 1.3 \times 10^{-4}\ s^{-1}$; *cf.* inset in Fig. 1). For a compilation of rate constants see Table 1.

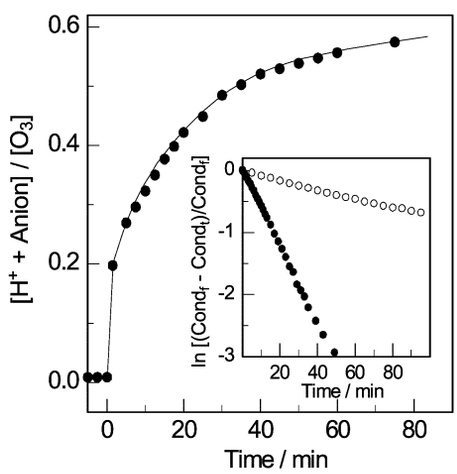



**Fig. 1** Ozonolysis of thymine in aqueous solution at 18 °C. Formation of acid (expressed as mol acid per mol ozone consumed) as a function of time as followed by conductance measurements. Inset: the slow part of conductance increase at 3 °C (○) and 18 °C (•) plotted as if it were of first-order kinetics.

The calibration of the conductometric set-up has been done with sulfuric acid. The acids that are formed in this system are weak acids (the acidic hydroperoxide **5** has a $pK_a$ of 4.0, see below, and formic acid one of 3.75) and are hence partially protonated ([ozone] = $2 \times 10^{-4}$ mol $dm^{-3}$ in these experiments). The "prompt" acid yield corrected for partial protonation is then calculated at ~34% (with respect to consumed ozone). Further acid is released in the slow process whereby its yield increases to ~75%.

Formic acid yields have been measured by ion chromatography. The formic acid yield measured about 5 min after ozonolysis is noticeably lower (~43%) than after ~100 min

**Table 1** Compilation of rate constants relevant to this study

| Reaction | Rate constant | Reference |
|---|---|---|
| Thymine + $O_3$ ⟶ products | $4.2 \times 10^4$ dm$^3$ mol$^{-1}$ s$^{-1}$ | Ref. 22 |
| | $3.4 \times 10^4$ dm$^3$ mol$^{-1}$ s$^{-1}$ | This work |
| Thymine anion + $O_3$ ⟶ products | $3 \times 10^6$ dm$^3$ mol$^{-1}$ s$^{-1}$ | Ref. 22 |
| Fast acid release | > 70 s$^{-1}$ | This work |
| Slow formic acid release, 18 °C | $1.1 \times 10^{-3}$ s$^{-1}$ | This work |
| Slow formic acid release, 3 °C | $1.3 \times 10^{-4}$ s$^{-1}$ | This work |
| Decay of **5**, 18 °C | $1.0 \times 10^{-3}$ s$^{-1}$ | This work |
| Slow $H_2O_2$ release, 18 °C | ~$1 \times 10^{-3}$ s$^{-1}$ | This work |
| **5** (and **6**) + $R_2S$ ⟶ products | 50 dm$^3$ mol$^{-1}$ s$^{-1}$ | This work |
| **5** (and **6**) + $I^-$ ⟶ products | 43 dm$^3$ mol$^{-1}$ s$^{-1}$ | This work |
| **18** + $I^-$ ⟶ products | 7.5 dm$^3$ mol$^{-1}$ s$^{-1}$ | This work |
| **5** (and **6**) + $Fe(CN)_6^{4-}$ ⟶ products | 0.4 dm$^3$ mol$^{-1}$ s$^{-1}$ | This work |
| **18** + $Fe(CN)_6^{4-}$ ⟶ products | 1 dm$^3$ mol$^{-1}$ s$^{-1}$ | This work |

**Table 2** Compilation of yields (with respect to ozone consumed) in the ozonolysis of thymine

| Process | Yield (%) | Reference |
|---|---|---|
| "Prompt" acid formation (conductometry) | ~34 | This work |
| Formic acid at ~100 min (conductometry) | ~75 | This work |
| Formic acid at ~100 min (IC) | 75 | This work |
| Formic acid at high pH (IC) | 100 | This work |
| Acetic acid | Absent | This work |
| Total hydroperoxide (immediate) | 100 | This work |
| Total hydroperoxide (2 h) | 93 | This work |
| Hydrogen peroxide (immediate, catalase assay) | 25 | This work |
| Hydrogen peroxide (immediate, $R_2S$ assay) | 25 | This work |
| Hydrogen peroxide (after 1 h) | 40 | This work |
| Organic hydroperoxides (immediate) | 75 | This work |
| Organic hydroperoxides (after 1 h) | 53 | This work |
| 1-Hydroperoxymethylene-3-(2-oxopropanoyl)urea **5** | 34 | This work |
| 5-Hydroperoxy-5-methylhydantoin **18** (after 1 h) | 53 | This work |
| 5-Hydroxy-5-methylhydantoin **13** (after $R_2S$ treatment) | 67 | This work |
| 5-Hydroxy-5-methylhydantoin **13** (after $R_2S$ and $OH^-$ treatment) | 100 | This work |
| Singlet dioxygen (at high pH) | 8 | Ref. 29 |

(~75%). In neutral solutions, this value does not increase further with time. A treatment with base (KOH, pH 10.8, 90 min, 18 °C) brings the formic acid yield to 100%. For a compilation of yields see Table 2.

In order to test whether the prompt acid release can also be resolved kinetically, stopped-flow experiments with conductometric detection were carried out. As can be seen from Fig. 2, the build-up of conductance follows first order-kinetics, and the rate of reaction depends on the thymine concentration.

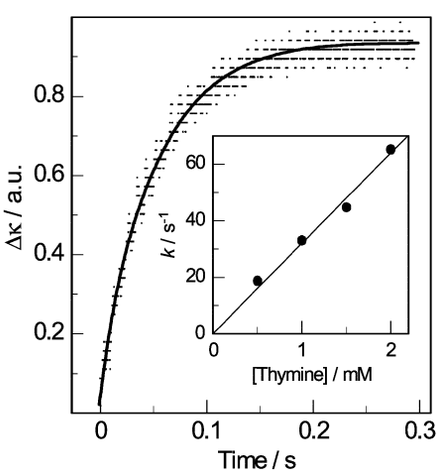



**Fig. 2** Ozonolysis of thymine in aqueous solution at 18 °C. Formation of an acid (assigned to **5**) in the reaction of ozone with thymine as followed by stopped-flow with conductometric detection. The solid line through the data points is a first-order fit. Inset: $k_{obs}$ as a function of the thymine concentration.

The rate constant derived from the data shown in the inset of Fig. 2 ($k = 3.4 \times 10^4$ dm$^3$ mol$^{-1}$ s$^{-1}$) agrees with the value obtained by competition kinetics ($4.2 \times 10^4$ dm$^3$ mol$^{-1}$ s$^{-1}$)[22] considering the errors involved in these measurements, especially in the value obtained by competition kinetics. It is thus concluded that in this fast conductance increase the rate-determining step is the reaction of ozone with thymine. It will be shown below that an acidic hydroperoxide, assigned to **5**, is formed. Compared to the reaction of ozone with thymine which gives rise to the thymine-derived Criegee ozonide, the decay of this Criegee ozonide (leading to **5**) and the subsequent deprotonation of **5** are fast.

**Formation and decay of hydroperoxides.** The total hydroperoxide yield is 100% as assayed with molybdate-activated iodide. Upon treatment of the ozonated solution with catalase, the hydroperoxide yield is reduced to 75%. Thus, $H_2O_2$ may be present right after ozonolysis in 25% yield. However, we have observed that formic peracid, a conceivable product in the present system, is also rapidly degraded by catalase,[30] and further experiments have been carried out to ascertain that the hydroperoxide that is eliminated by catalase is indeed $H_2O_2$. To this point, bis(2-hydroxyethyl) sulfide ($1 \times 10^{-3}$ mol dm$^{-3}$) has been added to the ozonated solution. This sulfide reacts fast with formic peracid ($k = 220 \times$ dm$^3$ mol$^{-1}$ s$^{-1}$)[30] yielding formic acid (*i.e.* leading to an increase in conductance, p$K_a$(HC(O)-OOH) = 7.1, p$K_a$(HC(O)OH) = 3.75), but no additional increase of conductance is observed. On the contrary, a decrease in the conductance occurs ($k = 50$ dm$^3$ mol$^{-1}$ s$^{-1}$), and the "slow" increase in conductance shown in Fig. 1 is also suppressed. This is taken as evidence that the observed "fast" formation of conductance is due to an acidic hydroperoxide (**5**, see below). The "slow" increase in conductance must also have

(formic acid releasing) hydroperoxides as precursors (**5** and **6**, suggested, see below), and formic peracid is not a product of the ozonolysis of thymine.

The reaction of bis(2-hydroxyethyl) sulfide cannot be used to determine the yield of reactive hydroperoxides, since its sulfoxide is difficult to detect by HPLC due to its low absorption coefficient in the accessible wavelength region. However, methionine also undergoes the corresponding reaction with reactive hydroperoxides, and its sulfoxide is more readily detected.[30] Using this assay, the yield of sulfide-reactive hydroperoxides is 75%. The complement to 100%, a value of 25%, has been determined by measuring the hydroperoxide yield remaining after the destruction of sulfide-reactive hydroperoxides with bis(2-hydroxyethyl) sulfide (Fig. 3). It has

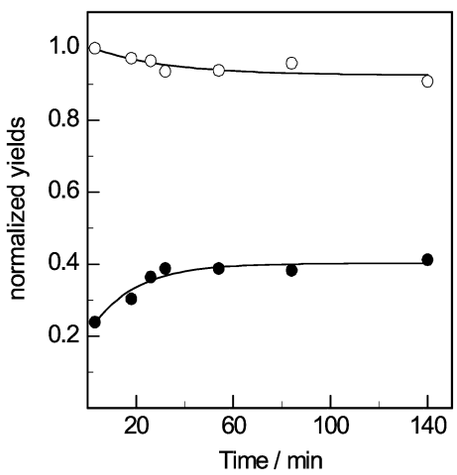



**Fig. 3** Ozonolysis of thymine. Normalised yields of total hydroperoxides (○) and hydrogen peroxide (•) as a function of time after ozonolysis. Hydrogen peroxide yields were determined after the organic hydroperoxides had been destroyed with sulfide.

been shown that $H_2O_2$ does not react with bis(2-hydroxyethyl) sulfide at an appreciable rate.[30] Based on the catalase and the sulfide assays, it is hence concluded that $H_2O_2$ is present in 25% yield right after ozonation.

Thus, at least three kinds of hydroperoxides are formed immediately upon ozonation, an acidic hydroperoxide (assigned to **5**, see below), a neutral hydroperoxide (**6**, suggested, see below) and $H_2O_2$. The organic hydroperoxide remaining one hour after ozonation is a further neutral hydroperoxide (assigned to **18**, see below).

As is seen from Fig. 3, the total hydroperoxide yield is not much (~7%) decreased over time. A fraction of the sulfide-reactive ones decays yielding partly $H_2O_2$. This reaction proceeds with a half-life of ~12 min according to a computer fit through the data (• in Fig. 3). Within error limits, it follows the same kinetics as the "slow" acid release reported above ($t_{1/2}$ = 10.5 min), *i.e.* a new organic hydroperoxide (assigned to **18**, see below) appears during the decay of the primary ones. The yield of $H_2O_2$ during this decay of the primarily formed hydroperoxides (**5** and **6**, see below) is much lower (~15%) than that of formic acid (~75%). The loss of total hydroperoxide yield (~7%) also occurs at the same time scale (*cf.* Fig. 3).

Hydrogen peroxide does not react with iodide at an appreciable rate unless activated by molybdate, but the more reactive hydroperoxides do. The hydroperoxides present right after ozonolysis (**5** and **6**, see below) react with iodide (without molybdate activation) more rapidly ($k$ = 43 dm$^3$ mol$^{-1}$ s$^{-1}$, determined by stopped flow) than the one remaining after one hour (**18**, for its identification see below; $k$ = 7.5 dm$^3$ mol$^{-1}$ s$^{-1}$). Accordingly, the apparent $k_{obs}$ of the reaction changes with time, since the rate constants of these hydroperoxides differ only by a factor of ~5.7, *i.e.* these two reactions are not well separated kinetically. The change in $k_{obs}$ with time shows a half-value of ~13 min (data not shown).

The reactivity of the two sets of organic hydroperoxides (immediate, **5** and **6**, and late, **18**) toward $Fe(CN)_6^{4-}$ is very similar. Right after ozonolysis and destruction of $H_2O_2$ with catalase, the rate constant of the then prevailing organic hydroperoxides **5** and **6** was $k$ = 0.4 dm$^3$ mol$^{-1}$ s$^{-1}$ and after keeping the samples for one hour to let **5** and **6** decay into **18**, the rate constant was $k$ = 1 dm$^3$ mol$^{-1}$ s$^{-1}$. When based on the iodide assay, **5** and **6** yield two mol $Fe(CN)_6^{3-}$, while **18** yields 2.9 mol. Fe(II)-based determinations of hydroperoxides often lead to higher than stoichiometric (2 mol Fe(III) per mol hydroperoxide) relationships (one electron reduction of organic hydroperoxides leads to the formation of alkoxyl radicals which may undergo fragmentation yielding alkyl radicals; subsequent $O_2$-addition and reduction gives rise to new hydroperoxides, *cf.* ref. 30). Interestingly, the reactivities of these two sets of hydroperoxides towards the two reductants studied here, iodide and $Fe(CN)_6^{4-}$, are reversed.

**HPLC and UV-spectroscopy.** Right after ozonolysis, a strongly UV-absorbing ($\lambda_{max}$ = 256 nm) product (Fig. 4, assigned

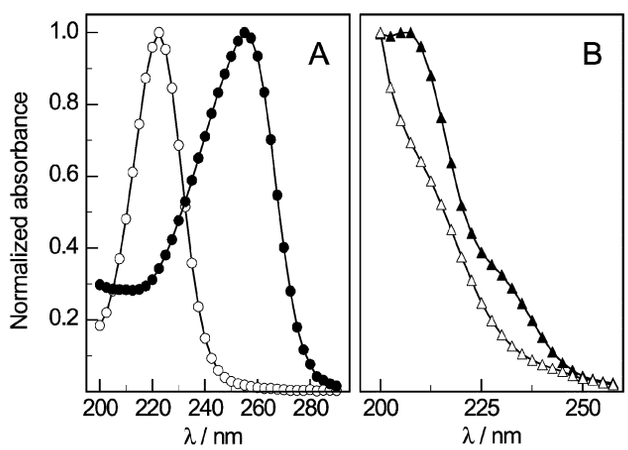



**Fig. 4** UV absorption spectra (and retention times with water as eluent) of the products formed in the ozonolysis of thymine. Frame A: •, the anion of 1-hydroperoxymethylene-3-(2-oxopropanoyl)urea **5a** (7.6 min); ○, 5-hydroperoxy-5-methylhydantoin **18** (6 min). Frame B: ▲, 1-formyl-5-hydroxy-5-methylhydantoin **11** (8.3 min); △, 5-hydroxy-5-methylhydantoin **13** (5.4 min, reference material available).

to **5a**, see below) is observed by HPLC on a reversed-phase column with a retention time of 7.6 min using water as eluent. There is also a second product (assigned to **11**, see below) eluting at 8.3 min. This has only an end-absorption tailing towards 220 nm. Both products must be quite polar, since they elute much earlier than thymine (15.6 min). As will be discussed below, we are forced to account for a further organic hydroperoxide (**6**, suggested). Compared to **5**, hydroperoxide **6** is expected to have a lower UV-absorption and may be masked if its retention time is very close to that of **5**.

When the pH of the eluent is decreased (with sulfuric acid), **5** elutes somewhat later with a concomitant shift of its absorption maximum towards 237 nm (*cf.* inset in Fig. 5). From the data shown in the main graph of Fig. 5, the p$K_a$ value of **5** is calculated at 4.0. For taking the spectra in Figs. 4 and 5, different set-ups (including columns) were used. It is noted that the spectrum of **5a** given in Fig. 5 lacks the short-wavelength absorption shown in Fig. 4. Possibly, on the column used for running the spectra shown in Fig. 5 the separation was better, and in Fig. 4 the spectrum of **5a** is contaminated by contributions of **6**. For this most likely weakly-absorbing hydroperoxide no UV-spectrum is available. The suggestion that **5** and **6** coelute is supported by post-column derivatisation with molybdate-activated iodide, where right after ozonolysis only two

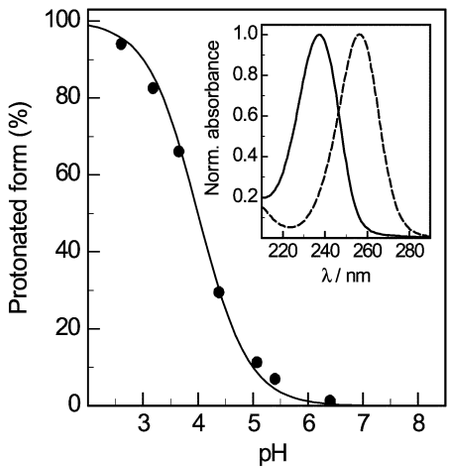



**Fig. 5** Percentage of undissociated 1-hydroperoxymethylene-3-(2-oxopropanoyl)urea **5** as a function of pH as obtained by HPLC. The pH was varied by varying the pH of the eluent. UV spectra of **5** (solid line: $\lambda_{max}$ = 237 nm, eluent pH 2.6) and its anion (**5a**, dashed line: $\lambda_{max}$ = 256 nm, eluent pH 7).

hydroperoxides, $H_2O_2$ and a rather broad peak due to organic hydroperoxide(s) (retention times under these conditions 7.6 min, 22% yield, and 9.7 min, 78% yield) were detected. One hour after ozonation the $H_2O_2$ peak has increased (39% of total hydroperoxide), and the peak of the organic hydroperoxide (61%, attributed to **18**) is now sharp and shifted to 9.5 min.

The hydroperoxide **5** decays by the same kinetics (Fig. 6,

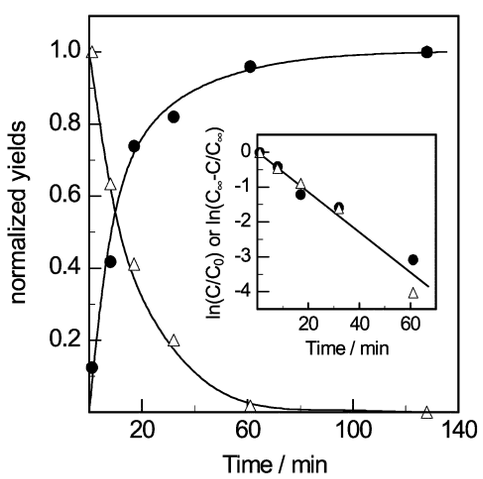



**Fig. 6** Ozonolysis of thymine. Decay of the anion of 1-hydroperoxymethylene-3-(2-oxopropanoyl)urea **5a** and build-up of 5-hydroperoxy-5-methylhydantoin **18** as a function of time. Inset: first-order kinetic plots of the data.

inset: $k = 1.0 \times 10^{-3}$ s$^{-1}$) as found for the release of formic acid ($k = 1.1 \times 10^{-3}$ s$^{-1}$, see Table 1). It gives rise to the hydroperoxide **18** which elutes at 6.0 min. It absorbs at shorter wavelength ($\lambda_{max}$ = 220 nm) than its precursor **5** (*cf.* Fig. 4).

The hydroperoxides **5** and **18** are both wiped out upon the addition of bis(2-hydroxyethyl) sulfide which shows that they must be strongly oxidising hydroperoxides (*cf.* ref. 30). The hydroperoxide **5** (together with **6**, see below) is reduced to **11**, while **18** is reduced to **13**. When the sulfide was added one hour after ozonation (to let the primary hydroperoxides (**5** and **6**) decay into **18**), the yield of 5-hydroxy-5-methylhydantoin **13** was 67% (reference material for the quantification of **13** was available). When this solution was subsequently treated with NaOH at pH 10.5 overnight, product **11** also disappeared, and the yield of 5-hydroxy-5-methylhydantoin **13** increased to ~100%. When the sulfide is added immediately after ozonolysis, 1-formyl-5-hydroxy-5-methylhydantoin **11** is the only observed product. As expected from the above, **11** does not lose formic acid at natural pH, *i.e.* it does not decay into **13** under these conditions.

The molar absorption coefficient of **5a** at 256 nm must be higher than that of thymine at the same wavelength. When equal volumes of thymine ($4 \times 10^{-4}$ mol dm$^{-3}$) and ozone ($2.25 \times 10^{-4}$ mol dm$^{-3}$) solutions were mixed, the absorption at 256 nm rose by a factor of 1.18 (taking the dilution by the ozone solution into account) and subsequently decayed again ($k = 1.0 \times 10^{-3}$ s$^{-1}$, see above) to a level which is compatible with the expected remaining thymine concentration and no significant absorption of the remaining products at 256 nm. Taking the yield of the 256 nm species as 34% (based on the immediate conductance formation, *cf.* Fig. 1), its molar absorption coefficient is calculated to be $2.5 \times 10^4$ dm$^3$ mol$^{-1}$ cm$^{-1}$, *i.e.* more than three times that of thymine at its maximum at 265 nm ($\varepsilon = 7.9 \times 10^3$ dm$^3$ cm$^{-1}$ s$^{-1}$).

**LCMS.** The LCMS-ESI technique in the positive mode was used for the determination of the molecular weights of some of the key products. This technique has the disadvantage that some compounds do not give rise to a pronounced spectrum even if present at a reasonably high concentration. Thus, they escape detection. Yet, some of the hydroperoxides were detectable. Some of these, *e.g.* **5**, rapidly decay at room temperature. At 3 °C, the decay of **5** is much slower ($1.3 \times 10^{-4}$ s$^{-1}$, *cf.* inset in Fig. 1). This eight-fold prolonged lifetime enabled us to carry out LCMS measurements with a sample that had been ozonated at 3 °C. A peak at $m/z$ = 175, $(M + H)^+$, was observed indicating that the molecular weight of **5** is 174 Da, *i.e.* it contains three more oxygens compared to thymine. The mass spectrum of **5** was weak, since even at 3 °C a considerable fraction of it had decayed. No mass spectral evidence was obtained for **6**, the other inferred primary organic hydroperoxide. Product **11**, the complement of $H_2O_2$ formed immediately upon ozonation (see below), does not give rise to a mass spectrum under these experimental conditions.

When **5** had decayed, the mass spectrum of **18** could be taken and showed a pronounced $m/z$ = 147, $(M + H)^+$, together with ~10% $m/z$ = 293 $(2M + H)^+$ *i.e.* its molecular weight is 146 Da. Similarly to product **11**, the final product 5-hydroxy-5-methylhydantoin **13** (authentic reference material was available) does not give rise to a mass spectrum under these conditions.

As mentioned above, **5** and **18** are hydroperoxides and are eliminated by the addition of bis(2-hydroxyethyl) sulfide, and as a consequence of this, compounds giving rise to an LCMS response are no longer present except for the resulting sulfoxide (MW = 138 Da) which shows a pronounced signal at $m/z$ = 139, $(M + H)^+$ together with some $m/z$ = 277 $(2M + H)^+$. The parent sulfide does not give an LCMS signal.

**Assignment of the products.** Product **5** has a molecular weight of 174 Da. It is a strongly oxidising hydroperoxide, *i.e.* the hydroperoxide function must be activated by electron-withdrawing groups. It is a fairly strong acid ($pK_a$ = 4.0). The spectral shift from 237 to 256 nm upon deprotonation requires a considerable conjugation of the π system. Its structure is assigned to 1-hydroperoxymethylene-3-(2-oxopropanoyl)urea.

Product **11** is not a peroxide. Upon hydrolysis, **11** is converted into 5-hydroxy-5-methylhydantoin **13** and formic acid. Hence, **11** is assigned to 1-formyl-5-hydroxy-5-methylhydantoin.

Product **18** is a hydroperoxide with the molecular weight of 146 Da. Its precursors are **5** and **6** (see below). Upon reduction of **18** with sulfide, 5-hydroxy-5-methylhydantoin **13** is formed. Hence, **18** is assigned to 5-hydroperoxy-5-methylhydantoin.

**Ozonolysis at high pH.** Thymine deprotonates at high pH ($pK_a$ = 9.9), and the rate of reaction with ozone becomes considerably faster ($k = 3 \times 10^6$ dm$^3$ mol$^{-1}$ s$^{-1}$).[22] Concomitantly, the reaction mechanism must be partially altered, since now the

formation of singlet dioxygen, $O_2(^1\Delta_g)$ is observed in 8% yield.[29] At pH 11.6, the formation of the 256 nm species **5** is no longer observed. This must be due to a change in reaction mechanism. Its formation would not have escaped our attention, since the kinetics of the decay of **5** at this pH is less than a factor of 2 faster than at pH 4 (shown by adjusting a thymine solution ozonated at natural pH to pH 11.6).

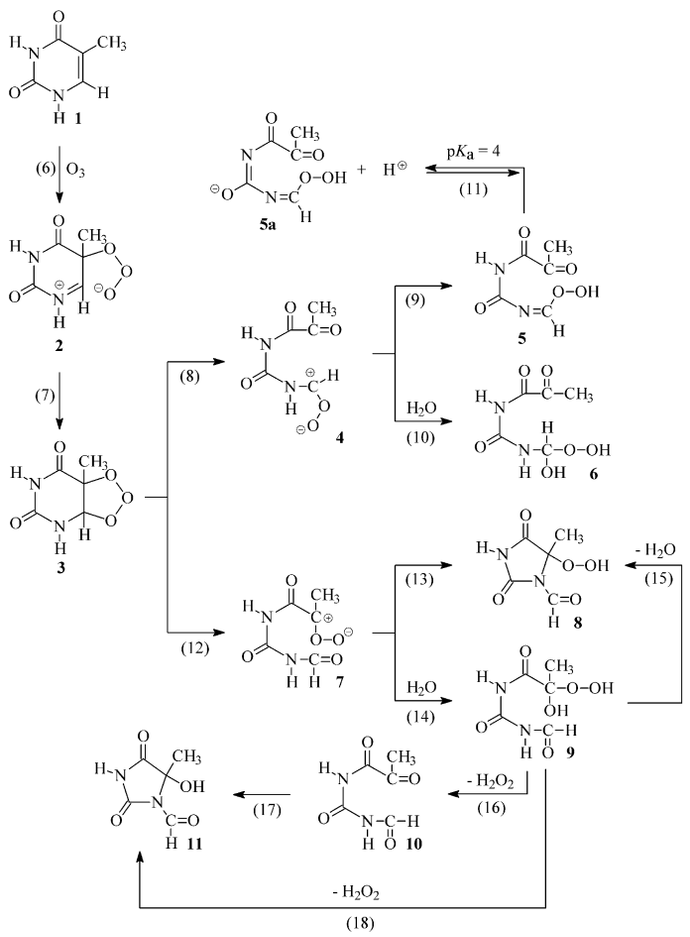
Thymine glycol is observed by GCMS after trimethylsilylation.

**Mechanistic aspects.** Ozone is a strongly electrophilic agent[31] and will hence preferentially add to the C(5)-position of thymine **1** [reaction (6)], as other electrophilic agents such as the $^{\bullet}OH$ radical and the $H^{\bullet}$ atom do (*cf.* ref. 7). The zwitterion **2** will close the ring thereby forming the Criegee intermediate **3** [reaction (7)]. The Criegee intermediate **3** can now open the ring in two directions [reactions (8) and (12)]. The zwitterion **4** formed in the predominating reaction (8) can eliminate the proton at the neighbouring nitrogen [reaction (9)]. Hydroperoxides have generally high $pK_a$ values [*cf.* $pK_a(H_2O_2)$ = 11.8; $pK_a$(HC(O)OOH) = 7.1], and the hydroperoxide function of **5** will thus remain protonated at a pH < 7. The acidity observed for **5** [$pK_a$(**5**) = 4.0] is thus due to a deprotonation at N(3) [reaction (11)]. In competition with reaction (9), the zwitterion **4** may react with water yielding the α-hydroxyalkyl hydroperoxide **6** [reaction (10)]. One also may consider that the zwitterion **4** may form an N(3)–C(6) bond upon losing the acidic proton at N(3). However, the strain exerted by the four-membered ring may not be much in favour of this reaction, and hence this possibility is not shown in the reaction scheme.

The hydroperoxide **5** has a conjugated π system and shows a strong absorption at 237 nm (*cf.* Fig. 5). The proton at N(3) is acidic [equilibrium 11, $pK_a$(**5**) = 4.0], and the absorption maximum is shifted to 256 nm upon deprotonation. The fast build-up of conductivity that occurs on the same time scale as the reaction of thymine **1** with ozone (Fig. 2) is due to the formation of **5a** and a proton. In the sequence of reactions that lead to **5a** and $H^+$, the rate-limiting step is the reaction of ozone with **1** [reaction (6)]. From the conductivity data shown in Fig. 1, the yield of **5** must be close to 34% of ozone consumed.

Subsequent hydrolytic processes lead to the release of formic acid ($t_{1/2}$ ≈ 10.5 min at room temperature) monitored by the "slow" increase in conductivity (Fig. 1). These conductivity data suggest that the combined yield of the formic acid-releasing hydroperoxides (attributed to **5** and **6**) is close to 75%, *i.e.* the yield of **6** is ~41%. The kinetics of this "slow" rise in conductivity is mirrored by a very similar kinetics of the disappearance of the optical absorption of **5/5a**. The fact that **5** has a $pK_a$ value of 4.0 and formic acid one of 3.75 warrants the presence of additional precursors of the formic acid that is released in the "slow" process. It is suggested that both **5** and **6** contribute here. The evaluation of build-up kinetics is fraught with considerable errors, especially when more than one species is involved.[32] When the individual rate constants are not drastically different, they cannot be disentangled, and the plots shown in the inset of Fig. 1 are mainly given to show the marked reduction in rate of hydrolysis upon reducing the temperature to 3 °C. This enabled us to run the mass spectra of the hydroperoxides by LCMS. The molecular weight of **5** is 174 Da, agreeing with mass spectral data. There is no mass spectral evidence for the formation of **6**, but in view of its low concentration at the time of measurement and the absence of any mass spectral response in the case of **11** and **13**, this lack of confirmation does not carry much weight and thus does not rule out the formation of **6**. After hydrolysis, a hydroperoxide with the molecular weight of 146 Da remains. It is assigned to **18**. A tentative suggestion as to the mechanism of the hydrolysis of **5** and **6** will be given below.

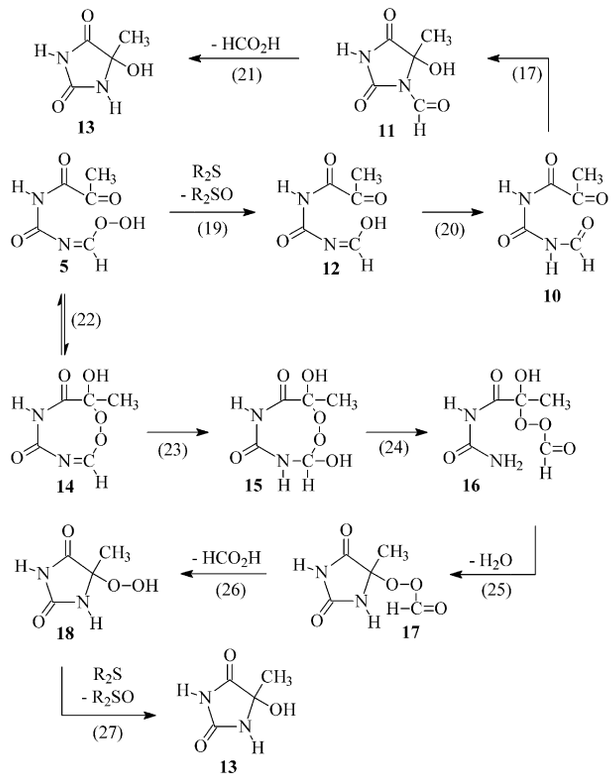
In competition with reaction (8), the Criegee intermediate **3** can decay according to reaction (12). The zwitterion **7** may give



rise to the hydroperoxides **8** and **9** [reactions (13)–(15)]. The hydroperoxide **9** is an α-hydroxyalkyl hydroperoxide. Such hydroperoxides often eliminate $H_2O_2$ so rapidly that it is not possible to determine their lifetime. A case in point is hydroxybenzyl hydroperoxide.[33] On the other hand, there are also α-hydroxyalkyl hydroperoxides such as hydroxymethyl hydroperoxide which have very long lifetimes in neutral solution and release $H_2O_2$ rapidly only at high pH.[34] The structural parameters that determine the stability of α-hydroxyalkyl hydroperoxides are as yet not known. We suggest that hydroperoxide **9** belongs to the unstable ones and that the $H_2O_2$ observed right after ozonolysis is due to reactions (16) and/or (18). The resulting product **10** will convert to 1-formyl-5-hydroxy-5-methylhydantoin **11**. This product is rather stable and releases formic acid only upon treatment at high pH. The yields of immediate $H_2O_2$ and formic acid released at high pH are both ~25%. It is thus suggested that reaction (14) dominates over reaction (13) and that reaction (15) is slow compared to reactions (16) and/or (18). However, the ozonolysis of thymidine which will be discussed below takes a very different route, and acetic acid is released from an intermediate analogous to **9**. No acetic acid is formed in the thymine system that we are concerned with here. This requires that in the thymine system there must be a much faster process competing with a potentially also possible acetic acid-releasing process. These two systems differ in the substitution at N(1). The rapid release of $H_2O_2$ in the thymine system may thus involve the N(1) proton. We thus suggest that $H_2O_2$ is released in the concerted pathway (18) which may well proceed with a relay of water molecules to accommodate a good transition state for the reaction to take place. In thymidine, the rate of acetic acid release is 0.55 $s^{-1}$ (see below). To compete with such a process effectively, the rate of reaction (18) must be considerably faster.

The reduction of hydroperoxide **5** by sulfide is depicted in reaction (19) [followed by reactions (20) and (17)] (hydroperoxide **6** will undergo analogous reactions), and since 1-formyl-5-hydroxy-5-methylhydantoin **11** eliminates formic acid only at high pH [reaction (21)], not only the conductance signal increase observed right after the addition of ozone (dissociation of **5**) but also the subsequent slow increase due to the release of formic acid disappears upon the addition of the sulfide. After hydrolysis at high pH, the only remaining product is 5-hydroxy-5-methylhydantoin **13** (100% after the addition of sulfide, reference material was available).

The "slow" release of formic acid with concomitant decay of hydroperoxide **5** and the other related hydroperoxide **6** may be rationalised by assuming that these hydroperoxides form α-hydroxyendoperoxides such as **14** [equilibrium (22)]. Hydroperoxides readily undergo such a reaction with carbonyl compounds. Often, the equilibrium constants are quite high, and the instability of α-hydroxyalkyl hydroperoxides [*cf.* reaction (16)] is then due to the inefficiency of the back-reaction at the low concentrations of the reactants (~$10^{-4}$ mol $dm^{-3}$) that we are concerned with here. This situation does not prevail in the intramolecular addition reaction (22). Addition of water to **14** leads to **15** [reaction (23)]. It is noted that upon endoperoxide formation, **6** immediately gives rise to **15**. It is important that in the next step no *N*-formyl compound is formed. This would not hydrolyse as readily ($t_{1/2}$ ≈ 13 min) as observed. Instead, we suggest reaction (24) which leads to the amide **16**. This may then undergo ring closure forming the hydantoin derivative **17** [reaction (25)] which subsequently hydrolyses [reaction (26)]. Mass spectral evidence for the formation of the hydroperoxidic hydantoin **18** has been given above. Its reduction by sulfide to 5-hydroxy-5-methylhydantoin **13** [reaction (27)] has been ascertained as well. It has been mentioned above that the decay kinetics of **5** and the build-up of formic acid are practically identical and do not deviate too much from a first-order rate law (*cf.* inset in Fig. 1). This could be accounted for if the rate



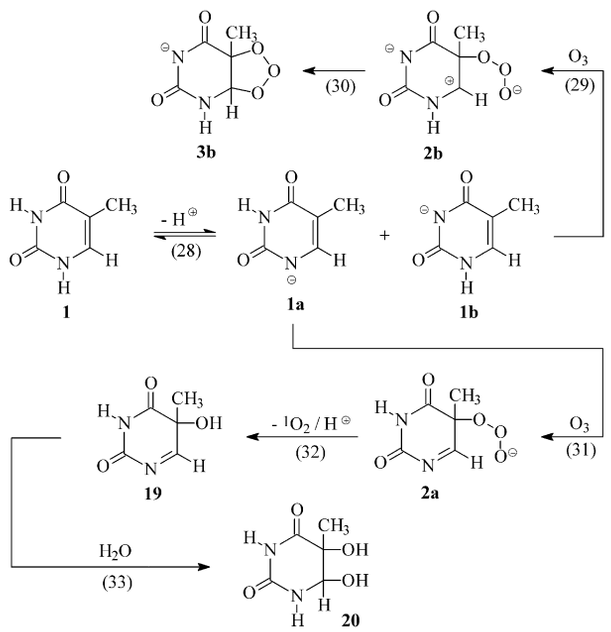

determining step in this sequence of reactions is the formation of the endoperoxide **14** (or **15** from **6**).

In the absence of sulfide, the hydroperoxides **5** and **6** mainly decay into the hydroperoxide **18** and formic acid. A small fraction also loses $H_2O_2$ in competition (*cf.* Fig. 3). Since the suggested mechanism involves an α-hydroxyalkyl peroxide as intermediate (**15** may convert into **9**), this is not unexpected. During this decay, the total hydroperoxide yield is also reduced to a small extent (7%). For this minor pathway that must lead to the formation of higher oxidised products, we do not present a mechanistic suggestion.

At high pH, thymine deprotonates [equilibrium (28), p$K_a$ = 9.9]. Since the acidity of N(1) and N(3) is very similar, anions **1a** and **1b** are both present at comparable concentrations.[35] It is tempting to predict that **1a** has a higher electron density in the C(5)–C(6) double bond which would favour reaction (31). However, thymidine which can only deprotonate at N(3) shows a similar increase in rate upon deprotonation as thymine. Hence, no prediction as to the preference of reaction (31) *vs.* reaction (29) can be made as yet.

The hydrotrioxide anion **2a** formed in reaction (31) has no positive charge at C(6) for a rapid ring closure to the Criegee intermediate. This will enhance the lifetime of this hydrotrioxide, *i.e.* it stands a chance of decomposing into **19** and singlet dioxygen, $O_2(^1\Delta_g)$ [reaction (32)].

Under these conditions the singlet dioxygen yield is 8%.[29] This value is low when compared with the yield of $O_2(^1\Delta_g)$ formation in the case of the 5-chlorouracil anion (~44%).[29] The reasons for this surprisingly low yield are not yet fully understood. Spin conversion and elimination of ground-state triplet dioxygen may compete. This process is energetically favoured by 105 kJ $mol^{-1}$. This effect is most prominent in the case of the reaction of $I^-$ and $Br^-$ with ozone.[30] In these systems, spin-orbit coupling *via* the heavy-atom effect favours singlet ⟶ triplet conversion, and singlet dioxygen formation is dramatically reduced. Here, where we compare thymine with 5-chlorouracil, this explanation does not hold, since it would only explain a reduced $O_2(^1\Delta_g)$ yield in the case of 5-chlorouracil. When a hydrotrioxide anion has a long lifetime and the energetics of singlet dioxygen elimination disfavour the reaction to proceed effectively, loss of ground-state triplet dioxygen has




been considered to proceed with quite some efficiency.[36] Furthermore, protonation of the hydrotrioxide anion by water may compete with its decay and other reactions of the hydrotrioxide may take place. Hydrotrioxides have often been assumed to be intermediates in the ozonation of aliphatic compounds,[37–39] but have recently been quite well characterised,[40] although their decay pathways are still not yet fully elucidated. It is an important observation that the decay of the hydrotrioxide derived from propan-2-ol is strongly catalysed by water.[40] On the singlet level, the water-catalysed decomposition of $H_2O_3$ is fairly well understood theoretically (and thus also that of organic hydrotrioxides).[41] Yet, singlet dioxygen was not detected in the ozonolysis of aqueous propan-2-ol solution at room temperature.[29] After a few seconds, no iodide or Fe(II) oxidising species was detectable (our own observations). Thus, it seems that water catalysis may have resulted in the release of ground-state (triplet) dioxygen. The present system is also poorly understood, and a more detailed product study than is presented here is highly desirable.

Product **19** formed in reaction (32) is an isopyrimidine species. Isopyrimidines are well-documented intermediates formed in the free-radical chemistry of uracil and its derivatives (*cf.* refs. 42,43). In the present system, addition of water to the N(1)–C(6) double bond will result in the formation of thymine glycol **20** [reaction (33)]. Its formation has been ascertained (see above), but no attempts have been made as yet to quantify this and other conceivable products.

### Thymidine

Thymidine reacts with ozone with a rate constant of $3 \times 10^4$ $dm^3$ $mol^{-1}$ $s^{-1}$ and its anion with a rate constant of $1.2 \times 10^6$ $dm^3$ $mol^{-1}$ $s^{-1}$.[22] In contrast to thymine, no singlet dioxygen is formed at high pH.[29] The products that have been reported in an earlier study[20] are also so markedly different from those reported above for the thymine reaction, that it seemed worthwhile to have a closer look at mechanistic details.

**Conductometry and ion chromatography.** Similarly to thymine, there is a fast and a slow build-up of conductance upon the addition of an ozone solution (Fig. 7).

In contrast to thymine, the fast process is two orders of magnitude slower (0.55 $s^{-1}$) than the reaction of thymidine with ozone under these conditions (60 $s^{-1}$). This indicates that this fast increase in conductance cannot be due to the formation of an acidic hydroperoxide (as **5** in the case of thymine). Besides

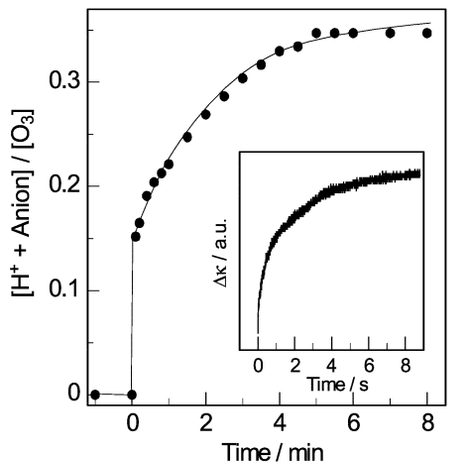



**Fig. 7** Build-up of conductance as a function of time in the reaction of thymidine ($8 \times 10^{-4}$ mol $dm^{-3}$) with ozone ($6 \times 10^{-5}$ mol $dm^{-3}$). Inset: the fast build-up resolved by the stopped-flow technique.

formic acid, acetic acid is formed here as well (the latter is absent in the case of thymine). The yield of this fast acid formation is ~18%, in agreement with the yield of acetic acid (see Table 3) when correction for the only partial dissociation of acetic acid ($pK_a = 4.8$) is made. Addition of sulfide 30 s after ozonation suppressed significantly the yield of formic acid but did not influence the acetic acid yield. It is thus concluded that acetic acid is released during this fast conductance rise.

The slower part of the conductance rise is due to the formation of formic acid, and its kinetics can be described by a first-order process ($k \approx 9 \times 10^{-3}$ $s^{-1}$). When the ozonated sample was kept for two hours at pH 11.6, the formic acid yield increased from ~76% to ~100%. The eluent used for IC ($1 \times 10^{-2}$ mol $dm^{-3}$ bicarbonate, pH 8.6) is slightly basic and thus labile formyl compounds may release formic acid upon chromatography beyond the amounts detected during the 8 min used to follow the conductance increase shown in Fig. 7 (main graph). For a compilation of yields see Table 3.

**Formation and decay of hydroperoxides.** When the molybdate-activated iodide reagent is added immediately, *i.e.* ~1–2 s after the addition of ozone, the total hydroperoxide yield thus determined is close to 100%. When the reagent is added after 25 s, only ~78% hydroperoxides are detected. Hence, there must be a very short-lived organic hydroperoxide with a yield of ~22%. An addition of catalase reduces the total hydroperoxide yield by 8%, *i.e.* only little $H_2O_2$ is formed. As determined by stopped-flow, the reactive organic hydroperoxide(s) present after 1 min react with iodide (without molybdate activation) with a rate constant of *ca.* 100 $dm^3$ $mol^{-1}$ $s^{-1}$, and after 8 min when most of the formic acid is released (*cf.* Fig. 7) a lower rate constant of around 45 $dm^3$ $mol^{-1}$ $s^{-1}$ is measured. After the rapid loss, the total hydroperoxide yield remains practically stable over time, and in contrast to thymine no $H_2O_2$ is released. Upon the addition of sulfide, the organic hydroperoxides are eliminated. The remaining hydroperoxide detectable with molybdate-activated iodide is $H_2O_2$ (confirmed by its kinetics).

**HPLC and LCMS.** Upon HPLC four major UV-absorbing peaks (retention times with water as eluent: 11.4, 12.8, 14.9 and 25.0 min; thymidine elutes at 45 min; $H_2O_2$ is not detectable by this method) disappeared upon sulfide addition and are thus attributed to reactive hydroperoxides. In contrast to thymine, an acidic hydroperoxide is not formed. Post-column derivatisation with molybdate-activated iodide monitors also weakly oxidising hydroperoxides such as $H_2O_2$ and allowed us to quantify their yields. Since a lower flow rate had to be used, the retention times are longer than those mentioned above (7.6 min, $H_2O_2$ (11.5%), 9.5 min (13.5%), 15.3 min (37%), 25.4 min (4%), 26.4

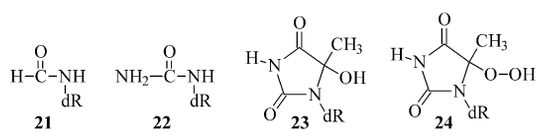
**Table 3** Compilation of yields (with respect to ozone consumed) in the ozonolysis of thymidine

| Process | Yield (%) | Reference |
|---|---|---|
| Acetic acid | 18 | This work |
| Fast acid release (as acetic acid, conductometry) | ~18 | This work |
| Acid release (8 min, as acetic plus formic acids, conductometry) | ~40–45 | This work |
| Formic acid (IC) | 76 | This work |
| Formic acid after 2 h at high pH (IC) | 100 | This work |
| Total hydroperoxide (immediate) | ~100 | This work |
| Total hydroperoxide (25 s–1.5 h) | 78 | This work |
| Hydrogen peroxide (25 s, catalase assay) | 8 | This work |
| Hydrogen peroxide (25 s, $R_2S$ assay) | 8 | This work |
| Hydrogen peroxide (after 1 h) | 8 | This work |
| Organic hydroperoxides (after 25 s) | 70 | This work |
| Organic hydroperoxides (after 1 h) | 70 | This work |
| $N_1$-(2-deoxy-β-D-erythropentofuranosyl)-5-hydroxy-5-methylhydantoin, two isomers **23** | 19.5 | Ref. 20 |
| $N_1$-(2-deoxy-β-D-erythropentofuranosyl)-5-hydroxy-5-methylhydantoin, two isomers **23** (after $R_2S$ treatment) | 43–50 | This work |
| *N*-(2-deoxy-β-D-erythropentofuranosyl)formamide **21** | 19 | Ref. 20 |
| *N*-(2-deoxy-β-D-erythropentofuranosyl)formylurea **22** | 18 | Ref. 20 |
| Singlet dioxygen (at high pH) | Absent | Ref. 29 |

min (4%), 29.9 min (3%), 32.5 min (5%)). The larger number of products as compared to thymine is largely due to the fact that now meso/(±) isomers are formed. In contrast to thymine, after five hours only small changes (retention times, number of peaks, yields) are observed in this HPLC chromatogram.

Upon LCMS of a sample ozonated at 3 °C, three species were detected with characteristic peaks at *m*/*z* = 162, *m*/*z* = 177 (together with 117 and 353) and *m*/*z* = 263 (together with 117). The latter peak was broad and is possibly due to two isomers. Upon reduction with bis(2-hydroxyethyl) sulfide the product characterised by *m*/*z* = 263 disappeared, and a new product with *m*/*z* = 247 (together with 117) is observed. Thus, the *m*/*z* = 263 species must be a hydroperoxide. The *m*/*z* = 117 peak in these mass spectra is typical of the 2-deoxyribosyl moiety, *i.e.* all these products retain the sugar moiety.

In the earlier study, the formation of *N*-(2-deoxy-β-D-erythropentofuranosyl)formamide **21** (MW = 161) has been reported and its yield determined at 19%.[20] This product seems to be present under our conditions as well (characterised by a prominent peak at *m*/*z* = 162, the M + 1 ion).




The *m*/*z* = 177 species is assigned to *N*-(2-deoxy-β-D-erythropentofuranosyl)urea **22**. Its molecular weight is 176, and the observed ion would be $(M + H)^+$. The also observed *m*/*z* = 353 is accounted for by the cluster ion $(2M + H)^+$. The previous study[20] does not report the formation of this product.

The *m*/*z* = 263 is due to a hydroperoxide. The primary hydroperoxide would have a molecular weight of 308 Da and should show an *m*/*z* = 309 $(M + H)^+$. Such a species is not observed, either because of its poor response or due to its rapid decay (considerable time after ozonolysis had elapsed before the mass spectra could be taken; the LCMS set-up was in a distant building). The molecular weight of the secondary hydroperoxide (loss of formic acid) $N_1$-(2-deoxy-β-D-erythropentofuranosyl)-5-hydroperoxy-5-methylhydantoin **24** is 262 Da, and we assign the observed 263 species to its $(M + H)^+$ ion. For this product, we would expect two isomers, and the broadness of the peak would account for this.

The *m*/*z* = 247 species is only observed after reduction with sulfide. Its precursor is the hydroperoxide **24**, and we attribute it to the $(M + H)^+$ ion of $N_1$-(2-deoxy-β-D-erythropentofuranosyl)-5-hydroxy-5-methylhydantoin **23** (MW = 246 Da). The formation of **23** has been reported before.[20]

Further species with weak signals are observed in this heavily ozonated sample, but it would be too speculative to try an assignment.

A few mg were isolated by preparative HPLC as reference material. After rotary evaporation, this material remained oily, and an HPLC chromatogram still showed some impurities. Using this material for calibration, the combined yield of the two hydantoins **23** was determined at 43%. Considering that the reference material contained impurities we also calculated their yield based on the assumption that they had the same molar absorption coefficient as their parent, 5-hydroxy-5-methylhydantoin **13** for which reliable material was available. We then obtain ~50%. In any case, the yield of **23** is much higher than the reported[20] value of only 19.5%. In the latter study,[20] the hydroperoxides were subjected without prior reduction to a laborious work-up, and possibly only a fraction underwent $H_2O_2$ elimination, while the rest was degraded.

**UV spectroscopy.** Equal volumes of thymidine ($2 \times 10^{-4}$ mol $dm^{-3}$) and ozone ($1.52 \times 10^{-4}$ mol $dm^{-3}$) solutions were mixed and the ensuing UV spectra run at 40 s and 10 min. From the measured UV spectra, the contribution of the remaining thymidine (now present at $4.8 \times 10^{-5}$ mol $dm^{-3}$) was subtracted. The difference spectra are shown in Fig. 8. While the species

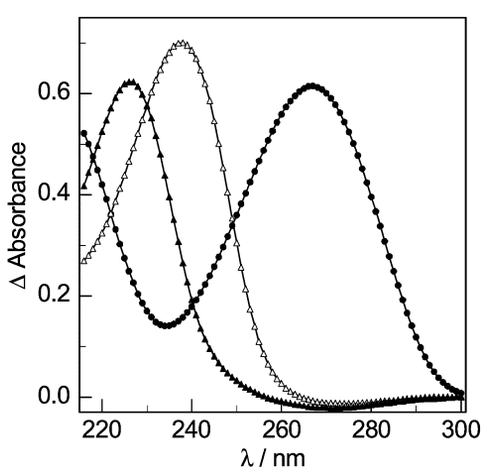



**Fig. 8** Thymidine ($7.6 \times 10^{-5}$ mol $dm^{-3}$) and product spectra after reacting equal volumes of thymidine (•, $2 \times 10^{-4}$ mol $dm^{-3}$) and ozone ($1.5 \times 10^{-4}$ mol $dm^{-3}$) solutions and subtracting the remaining thymidine. Spectra taken at ~40 s (Δ) and 10 min (▲). Running the spectra took 12 s.

with $\lambda_{max}$ = 238 nm decays, one with $\lambda_{max}$ = 226 nm builds up. The kinetics at these two wavelengths are the same ($k \approx 9 \times 10^{-3}$ $s^{-1}$). This value agrees with the release of formic acid (slow step in Fig. 7, main graph).

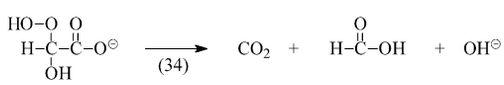
The same experiment as shown in Fig. 8 has been repeated by stopped-flow. There, the first observable product is also the species characterised by $\lambda_{max}$ = 238 nm. The kinetics of the spectra only reveal the development of two products. Right after ozonolysis the 238 nm species is present and decays following first-order kinetics ($k \approx 9 \times 10^{-3}$ $s^{-1}$) into the 226 nm species. Since the yields of the 238 nm and 226 nm species are not known, we are not able to give their molar absorption coefficients, but they must be > $10^4$ $dm^3$ $mol^{-1}$ $cm^{-1}$ at their maxima.

The kinetics that are observed by UV spectroscopy are paralleled by the slow conductance rise (*cf.* Fig. 7) which is due to formic acid release. The process observed by fast conductometry ($k = 0.55$ $s^{-1}$) is not detected by UV spectroscopy.

**Mechanistic aspects.** Although there are considerable similarities between thymine and thymidine in their reactions with ozone, there are also marked differences. In the case of thymine, the total hydroperoxide yield is 100% and decreases very little (by 7%) over more than two hours. With thymidine, the hydroperoxide yield is close to 100% only right after ozonolysis, but already after 25 s it has dropped to ~78%. Formation of $H_2O_2$ is important (25%) with thymine, but minor (8%) with thymidine. In thymidine, there is a rapid (0.55 $s^{-1}$) release of acetic acid (18%), a product that is not formed in the case of thymine. An acidic hydroperoxide such as **5** (34% in the case of thymine) is not formed with thymidine. Common to both systems is the full yield (~100%) of formic acid after reduction of hydroperoxides with sulfide and treatment at high pH. Yet, while 5-hydroxy-5-methylhydantoin **13** then reaches 100% in the case of thymine, the corresponding thymidine product **23** is only formed in a 43–50% yield. Much of this deficit can be accounted for by fragment products such as *N*-(2-deoxy-β-D-erythropentofuranosyl)formamide **21** and *N*-(2-deoxy-β-D-erythropentofuranosyl)urea **22**. A major problem in presenting a somewhat detailed mechanism for thymidine ozonolysis, is the lack of a material balance. It has already been pointed out above that we also cannot rely on the yields presented in the earlier study,[20] since considerable degradation of hydroperoxidic material must have occurred upon work up. Mechanistically, they[20] account for the formation of the $N_1$-(2-deoxy-β-D-erythropentofuranosyl)-5-hydroxy-5-methylhydantoins **23** which are their (and also our) major products by an elimination of $H_2O_2$. However, $H_2O_2$ is only formed in 8% yield (Table 3), and **24** is stable for hours (unless subjected to severe chromatographic conditions). In the earlier study,[20] it has also not been realised that acetic acid is a major product (18%). Especially the formation of the latter is a kind of key to mechanistic differences between thymine and its nucleoside.

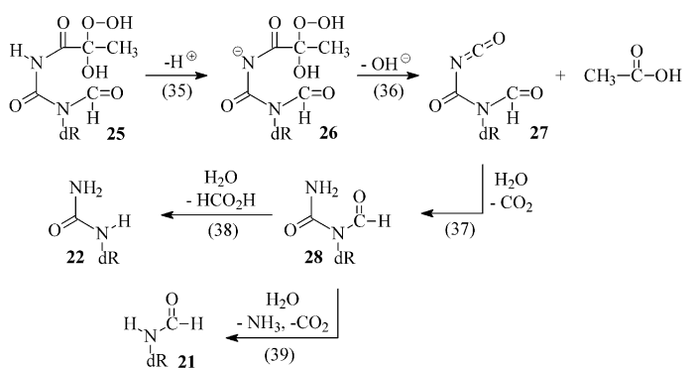
It is reasonable to assume that in both systems ozone attack will be preferentially at C(5) with the subsequent formation of the Criegee ozonide [*cf.* reactions (6) and (7)]. Subsequent ring opening according to the major pathway [*cf.* reaction (8)] leads to a zwitterion which only can deprotonate and yield a C–N double bond, when the nitrogen carries a hydrogen as substituent (as in thymine). Since in thymidine N(1) carries the 2-deoxyribosyl group instead, deprotonation and concomitant formation of an acidic hydroperoxide such as **5** is no longer possible. Here, the reaction analogous to reaction (10) that would lead to 2-deoxyribosyl-substituted **6** is the most likely one to occur. For this species we do not have mass spectral evidence, but it is tempting to attribute the 238 nm species to this intermediate. The subsequent formation of formic acid, *cf.* Fig. 7, will lead to **24** *via* an endoperoxide (*cf.* **6** ⟶ **15** ⟶ **16** ⟶ **17** ⟶ **18**) as depicted for the aglycon in reactions (25)–(27). The release of formic acid which occurs at the same rate as the decay of the 238 nm species could account for the formation of $N_1$-(2-deoxy-β-D-erythropentofuranosyl)-5-hydroperoxy-5-methylhydantoin **24** and the latter may thus have an absorption maximum at 226 nm. This is in agreement with the UV spectra of the hydroperoxides detected by HPLC. For **24** there is also mass spectral evidence (see above).

For the minor pathway of the decay of the Criegee ozonide shown for thymine as reaction (12) the fast hydantoin formation and concomitant release of $H_2O_2$ does not take place due to the lack of the hydrogen at N(1) (see above). Instead, acetic acid is released. The elimination of acetic acid with a concomitant drop in the total hydroperoxide yield is reminiscent of the rapid[33] decay of 2-hydroperoxy-2-hydroxyacetic acid [reaction (34)].




Here, we suggest that the primary hydroperoxide **25** can deprotonate at N(3) [reaction (35)], the N(3)H is acidified by two carbonyls in the α-position and further electron-withdrawing groups in the β-position, and that the ensuing anion undergoes the fragmentation reaction (36). The rate of acetic acid release is 0.55 $s^{-1}$ under the conditions of Fig. 7. The product of reaction (36), **27**, will be unstable and hydrolyse thereby eliminating $CO_2$ [reaction (37)]. The product that is formed in this reaction, *N*-(2-deoxy-β-D-erythropentofuranosyl)-*N*-formylurea **28**, as well as its further degradation product *N*-(2-deoxy-β-D-erythropentofuranosyl)formamide **21** [*cf.* reaction (39)], has been reported to be formed in high yield (*cf.* Table 3).[20] Above, it has been suggested that *N*-(2-deoxy-β-D-erythropentofuranosyl)urea **22** [*cf.* reaction (38)] may also be among the products.

The sum of the reported yields[20] of **21** and **28** is too high (37%) to balance the formation of acetic acid (18%, this work). Thus, they must be formed from other precursors (*e.g.* **24**) during work-up as well. If *N*-formylurea is a good model for **28**, the latter must hydrolyse rather slowly (at natural pH, 0.1 M *N*-formylurea releases only 0.04% formic acid within two hours.



As the pH is lowered, hydrolysis becomes progressively faster). The relatively high yield of **21** (19%)[20] may nevertheless have been formed during the laborious work-up, but degradation processes of labile (peroxidic) intermediates that have as yet not been revealed may contribute as well.

## Conclusion

Although the ozonolyses of thymine and thymidine have many mechanistic aspects in common, there is a noticeable influence of the substituent at N(1) on the pathways taken beyond the formation of the Criegee ozonide. In thymine, the Criegee ozonide opens into an intermediate that is capable of deprotonation at the neighbouring N(1)H. This new kind of reaction in ozone chemistry is not available to thymidine due to the lack of a hydrogen at this position. There are also major differences in the minor pathway. While acetic acid is released in the case of thymidine (18%), no such process occurs with thymine. Concerning ozone-induced DNA damage, it may be of importance that highly reactive hydroperoxides are formed which may lead to further DNA lesions, *e.g.*, upon reaction of these hydroperoxides with transition metal ions (*cf.* Fenton-type reactions).[44] On the other hand, these hydroperoxides might be readily destroyed by a sulfur compound such as gluthathione which is quite abundant (near millimolar)[45] in cells. In eukariotic cells where it is difficult to rationalise how ozone may reach the nucleus, DNA lesions such as 8-hydroxyguanine[9] may even be caused by hydroperoxidic intermediates generated in the reaction of ozone with cellular components other than DNA. Certainly, there must be a long chain of events from the first reaction of ozone with cellular components to the dramatic morphologic alterations observed,[46] *e.g.*, in cells of the lung exposed to ozone.

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