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Degradation of Nucleic Acids with Ozone. V. Mechanism of Action of Ozone on Deoxyribonucleoside 5′-Monophosphates[1]

KOZO ISHIZAKI,[a] NARIKO SHINRIKI,*[,a] and TOHRU UEDA[b]

Government Industrial Development Laboratory, Hokkaido,[a] Tsukisamu-Higashi 2–17, Toyohira-ku, Sapporo 061–01, Japan and Faculty of Pharmaceutical Sciences, Hokkaido University,[b] Kita-ku, Sapporo 060, Japan

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The degradation of 2′-deoxycytidine 5′-monophosphate (dCMP), 2′-deoxythymidine 5′-monophosphate (dTMP), 2′-deoxyadenosine 5′-monophosphate (dAMP) and 2′-deoxyguanosine 5′-monophosphate (dGMP) with ozone in aqueous solution was investigated. The patterns of degradation were essentially the same as those of the corresponding ribonucleotides. The second-order rate constants of the reactions as determined by stopped-flow spectrophotometry at pH 6.9 and 15 °C were $1.6 \times 10^4\,\text{M}^{-1}\,\text{s}^{-1}$ and $1.4 \times 10^3\,\text{M}^{-1}\,\text{s}^{-1}$ for dTMP and dCMP, respectively. The rate constants for dGMP and dAMP as estimated by a competitive technique under the same conditions were about $5 \times 10^4\,\text{M}^{-1}\,\text{s}^{-1}$ and $2 \times 10^2\,\text{M}^{-1}\,\text{s}^{-1}$, respectively. The pH dependencies of the relative rate constants, k_{rel}, of nucleotide pairs as well as the effect of a radical scavenger on k_{rel} suggested that the predominant reaction pathway is the direct attack of ozone on the base moiety in the cases of dCMP, dTMP and dGMP. On the other hand, radical reactions with the sugar moiety seemed to be predominant in the case of dAMP.

Keywords——ozone; ozonization; dAMP; dGMP; dTMP; dCMP; ozonization rate constant

The effects of ozone on biochemical systems have been extensively studied in recent years, including work on aberrations of chromosomes of human cells[2] and mutational changes of *Escherichia coli*[3] caused by the exposure of ozone. Since Brinkman *et al.* have observed that the effect of ozone on the deoxygenation of oxyhemoglobin in human blood was similar to that of ionizing radiation (·OH radical reaction),[4] a radiomimetic mechanism has been suggested for ozone reactions.[5] In addition, the action of bleomycin on deoxyribonucleic acid (DNA) was reported to involve hydrogen abstraction from the C4′ position of the deoxyribose moiety, resulting in strand scission, by a radical species such as ·OH.[6] Our recent results on ozone reactions with ribonucleotides and ribonucleic acids[7] suggested that direct (or ionic) ozone attack on the base moiety rather than a radical reaction is the predominant degradation pathway. It would be interesting to know whether or not the mode of action of ozone on DNA is different from that on ribonucleic acid (RNA). In the present study, the kinetics of ozone reactions with deoxyribonucleotides were examined in order to elucidate the degradation pathways.

Materials and Methods

Materials——Deoxyribonucleoside 5′-monophosphates were obtained from Sigma Chemical Co. All other chemicals used were of reagent grade.

Degradation of Deoxyribonucleotides with Ozone——Aqueous deoxyribonucleotide solutions (pH 7.8, 0.05 M H_3BO_3–NaOH) were bubbled through with ozone–oxygen gas mixture (gas flow rate, 330 ml/min; ozone concentration, 2 mg/l) in a 200 ml glass reactor at room temperature (*ca.* 22 °C). These reaction conditions were the same as those used for ribonucleotides.[7a)]

Stoichiometric Determination——Nucleotide solutions (pH 6.9 with 40 mM phosphate) were bubbled through with ozone–oxygen gas, and the material balances of nucleotide and ozone were determined. The ozone concentration in the gas introduced into the reactor was monitored by a spectrophotometer at 254 nm using a 10 cm flow cell.

The ozone remaining in the reactor effluent gas was absorbed in potassium iodide solution and determined iodometrically.[8a)]

Determination of Reaction Rate Constants——The second-order rate constants of the reactions in neutral solution were determined by using a stopped-flow spectrophotometer (Union Giken model RA-1200). Pseudo-first-order reaction conditions could not be employed in the present kinetic study due to the overlap of the ultraviolet (UV) absorption spectra of ozone and nucleotide. Therefore, the reactions were carried out under conditions such that the nucleotide concentration was slightly greater than the ozone concentration. The data were analyzed according to a second-order reaction equation.

For a reaction corresponding to Eq. 1,

$$\mathrm{pRN}+\mathrm{O_3} \longrightarrow \text{products} \qquad (1)$$

the second-order reaction equation is expressed as

$$\frac{1}{[\mathrm{pRN}]_0-[\mathrm{O_3}]_0}\ln\frac{[\mathrm{O_3}]_0[\mathrm{pRN}]}{[\mathrm{pRN}]_0[\mathrm{O_3}]}=kt. \qquad (2)$$

where pRN and k stand for a nucleotide and the second-order rate constant, respectively. For a light path of 1 cm, the absorbance, A, is given by

$$A=\varepsilon_{\mathrm{pRN}}[\mathrm{pRN}]+\varepsilon_{\mathrm{O_3}}[\mathrm{O_3}] \qquad (3)$$

where ε is the absorption coefficient. From the stoichiometric relationship,

$$[\mathrm{pRN}]_0-[\mathrm{pRN}]=[\mathrm{O_3}]_0-[\mathrm{O_3}] \qquad (4)$$

Equation 2 becomes

$$\ln\frac{A+\varepsilon_{\mathrm{O_3}}C_0}{A-\varepsilon_{\mathrm{pRN}}C_0}=C_0kt+\ln\frac{[\mathrm{pRN}]_0}{[\mathrm{O_3}]_0} \qquad (5)$$

where $C_0=[\mathrm{pRN}]_0-[\mathrm{O_3}]_0$. The rate constant, k, was determined by fitting Eq. 5 to the absorbance–time data.

The kinetic experiments were conducted by mixing the nucleotide solution with ozone solution at 15 ± 1 °C. The pH of the reaction mixture was adjusted to 6.9 by the addition of 80 mM phosphate to the nucleotide solution. The change in absorbance was followed at $\lambda_{\max}$ of the nucleotide. The reported values of $\varepsilon_{\mathrm{pRN}}$[9)] were used for each nucleotide. The values of $\varepsilon_{\mathrm{O_3}}$ at various wavelengths were determined by a modification of the procedure of Hoigné *et al.*[10)] By means of this procedure, $\varepsilon_{\mathrm{O_3}}$ at the $\lambda_{\max}$ of 260 nm was 2930 $\mathrm{M}^{-1}\,\mathrm{cm}^{-1}$. This value is in agreement with that reported.[11)] The stock solution of ozone was prepared by bubbling ozone–oxygen mixture through distilled water in an ice-water bath. The ozone concentration in the solution was determined spectrophotometrically immediately before the experiments.

Determination of Relative Rate Constants——Relative ozonization rates of the four nucleotides were determined by the competitive technique.[10,12)] When two nucleotides react with ozone in competition, the relative rate constant, k_{rel}, is given by Eq. 6,

$$k_{\mathrm{rel}}=\frac{k_2}{k_1}=\frac{\log[\mathrm{pRN_2}]_0/[\mathrm{pRN_2}]}{\log[\mathrm{pRN_1}]_0/[\mathrm{pRN_1}]} \qquad (6)$$

where k_1 and k_2 stand for the rate constants of the reactions of nucleotide 1 and nucleotide 2 with ozone, respectively. Equation 6 is derived on the assumption that each reaction is first-order in both ozone and nucleotide and that no other side reactions consuming ozone occur. Experimental procedures were basically the same as those of Williamson *et al.*[12)] A 5 ml buffered solution containing two nucleotides was placed into a test tube, and 2 ml of ozone solution was added, and mixed rapidly. The initial concentrations of each nucleotide and ozone in the reaction mixture were 0.20—0.30 mM and 0.15—0.25 mM, respectively. The total nucleotide concentration was always in excess of the ozone concentration. The k_{rel} values were determined in the pH range of 2.5—12. The pH was adjusted by adding phosphoric acid, sodium phosphate and sodium hydroxide. All reactions were carried out at 15 ± 0.5 °C.

Analysis——Deoxyribonucleotides were analyzed by high performance liquid chromatography using a $\mathrm{pNH_2}$-10 column (Shimadzu Seisakusho, Ltd.). Analytical procedures for sugars[7a,13)] and inorganic phosphate[8b)] are described elsewhere.

Results

Degradation of Deoxyribonucleotides with Ozone

The time courses of degradation of 2′-deoxycytidine 5′-monophosphate (dCMP), 2′-

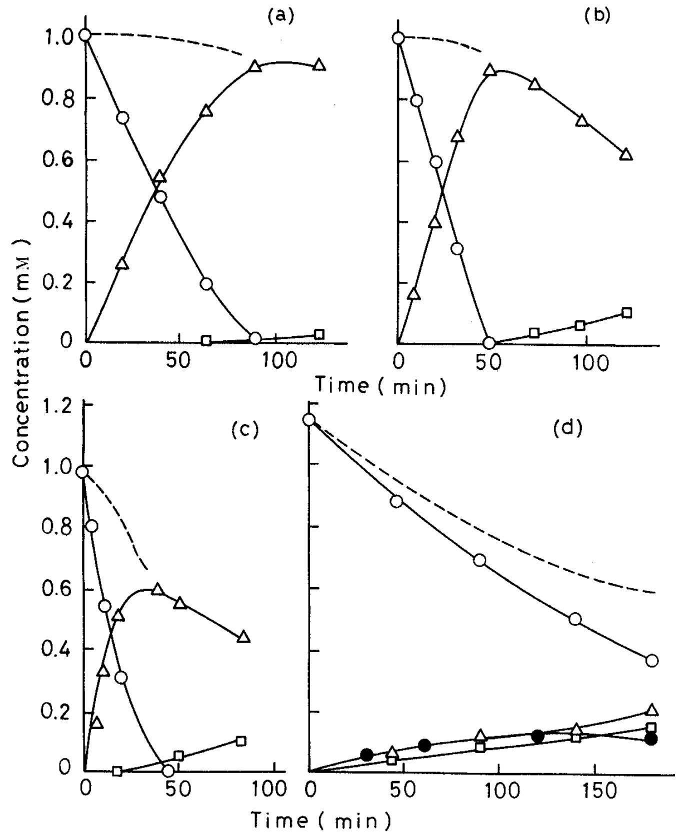

Fig. 1. Degradation of dCMP (a), dTMP (b), dGMP (c) and dAMP (d) with Ozone at pH 7.8

○—○, residual nucleotide ([pRN]); △—△, deoxyribose moiety except for that included in residual nucleotide ([pRN′][14]); ----, total sugar ([pRN]+[pRN′]); ●—●, free adenine; □—□, free phosphate ([PO_4^{3-}]).

deoxythymidine 5′-monophosphate (dTMP), 2′-deoxyguanosine 5′-monophosphate (dGMP) and 2′-deoxyadenosine 5′-monophosphate (dAMP) are shown in Fig. 1. It is apparent that the patterns of degradation of dCMP, dGMP and dAMP are analogous to those[7a] of the corresponding ribonucleotide. The pattern of degradation of dTMP was analogous to that of uridine 5′-monophosphate (UMP), although the former was degraded faster than the latter.

In the ozonization of dCMP and dTMP, both the degradation of the sugar moiety and the liberation of inorganic phosphate hardly occurred until the parent nucleotide had almost disappeared. The degradation of the base and sugar moieties of dGMP occurred concurrently, although the base was degraded much faster. dAMP was degraded in the same way as adenosine 5′-monophosphate (AMP), at the sugar moiety more than at the base. It should be pointed out, however, that dAMP was degraded significantly more slowly than AMP (the half-life for dAMP was about 40% longer than that for AMP).

Stoichiometry

The results of the stoichiometric determination are shown in Fig. 2. The stoichiometries (Δnucleotide/Δozone) of the reaction of dCMP, dTMP and dGMP were close to unity. However, the ratio was much less than unity in the case of dAMP.

Second-Order Rate Constants

The stopped-flow method was applicable only to the reactions of dCMP and dTMP. The ozonization products of dAMP and dGMP interfered with the spectrophotometric monitoring of the reactions, while there was no interference in the cases of dCMP and dTMP. Figure 3

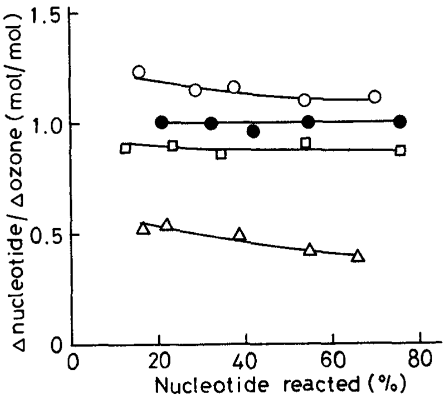

Fig. 2. Observed Stoichiometries of the Nucleotide–Ozone Reactions at pH 6.9

□—□, dCMP; ●—●, dTMP; ○—○, dGMP; △—△, dAMP.

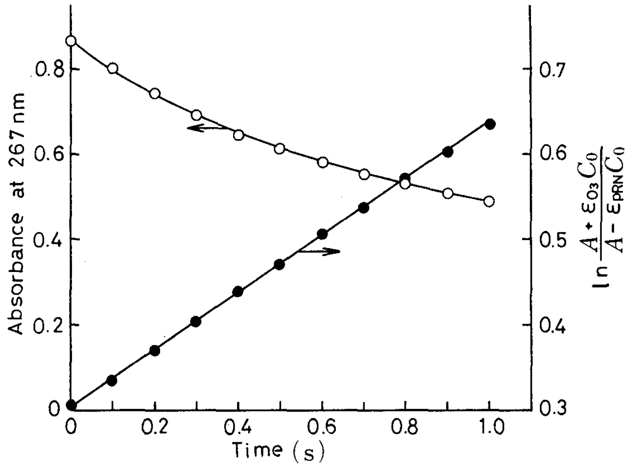

Fig. 3. Change in Absorbance (○) and Second-Oder Plot (●) of the dTMP–Ozone Reaction at pH 6.9 and 15 °C

$[\text{dTMP}]_0 = 0.080\,\text{mM}$; $[\text{O}_3]_0 = 0.059\,\text{mM}$.

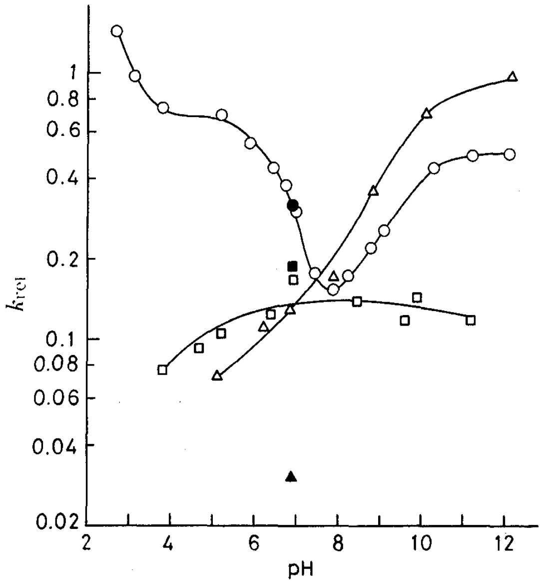

Fig. 4. Variations of Relative Rate Constants, k_{rel}, with pH at 15 °C

○—○, dTMP/dGMP; ●, dTMP/dGMP with 100 mM *tert*-butanol; □—□, dCMP/dTMP; ■, dCMP/dTMP with 100 mM *tert*-butanol; △—△, dAMP/dCMP; ▲, dAMP/dCMP with 100 mM *tert*-butanol.

Table I. Second-Order Rate Constants of the Reactions of Ozone with Deoxyribonucleoside 5′-Monophosphates and Nucleobases

Compound	k $\text{M}^{-1}\text{s}^{-1}$
dTMP	1.6×10^4
dCMP	1.4×10^3
Thymine	2.3×10^4
Cytosine	0.93×10^3

pH, 6.9; temp., 15 ± 1 °C; $[\text{nucleotide}]_0 = 0.10-0.06\,\text{mM}$; $[\text{base}]_0 = 0.10-0.06\,\text{mM}$; $[\text{O}_3]_0 = 0.05-0.07\,\text{mM}$

shows the absorbance change in the dTMP–ozone reaction. The second-order plot according to Eq. 5 was linear until the residual ozone fell to 30—40% of its initial concentration. The second-order plot for the dCMP reaction was also linear (data not shown). The rate constants, k, were calculated from the slope of the linear portion of the plots, and are shown in Table I. Each value of k is the average of four or five experiments. For comparison, the second-order rate constants of ozonization of cytosine and thymine were also determined (Table I).

Relative Rate Constants

The relative rate constants, k_{rel}, were determined to estimate the reaction rates of dGMP and dAMP with ozone. The k_{rel} values of three nucleotide pairs in the pH range 2.5—12 are shown in Fig. 4. As expected from Eq. 6, there is a large uncertainty in the determination of small k_{rel} values. Therefore, k_{rel} values of less than 0.1 may have large errors.

From the k_{rel} of the dTMP/dGMP pair and the rate constant of the dTMP reaction presented in Table I, we can roughly estimate the rate constant of the dGMP reaction at pH 6.9 and 15 °C as $5 \times 10^4 \mathrm{M}^{-1} \mathrm{s}^{-1}$. The rate constant of the dAMP reaction can also be approximated as $2 \times 10^2 \mathrm{M}^{-1} \mathrm{s}^{-1}$. The k_{rel} of the dCMP/dTMP pair at pH 6.9 in Fig. 4 is about 0.12. This value is in reasonably good agreement with that of 0.09 calculated from the data in Table I.

In order to examine the contribution of free radicals to the reactions, the effect of *tert*-butanol, a radical scavenger, was investigated at pH 6.9. As can be seen in Fig. 4, the addition of up to 100 mM *tert*-butanol has no appreciable effect on the k_{rel} of dTMP/dGMP (●) and dCMP/dTMP (■), while the k_{rel} of dAMP/dCMP (▲) is decreased significantly.

Discussion

The present results indicate that the change in the sugar moiety has no significant effect on the reactions of dCMP, dTMP and dGMP, thus suggesting that the sites of ozone attack are primarily on the base moieties. Ozone has been reported to attack the 5,6-double bond in certain pyrimidine bases and the 4,5-double bond in certain purine bases.(15) However, adenine is very stable as compared with guanine.(7a) Therefore, the slower degradation rate of dAMP than of AMP may be attributed to the relative stability of the 2′-deoxyribose moiety as compared to ribose.

Few kinetic data are available on ozone reactions in aqueous solution. Very recently Hoigné *et al.*(16) reported the ozonization rate constants of a wide variety of organic compounds other than nucleobases. The present work presents the first kinetic data on the ozonization of nucleotides. Deoxyribonucleotides (except adenine nucleotides) belong to the class of compounds considerably reactive with ozone.

It has been believed that the ozone reaction in aqueous solution consists of direct attack of ozone and free radical attack (*e.g.* by ·OH which is formed by the hydroxide ion-catalyzed decomposition of ozone).(10,17) Hoigné *et al.*(10) have observed that, in many ozonization systems containing two solutes, the relative rate constants reached around unity with increasing pH. These results can be explained by assuming that with increasing pH, the radical reactions (with less substrate–specificity) predominate over the highly substrate–specific direct ozonizations.

The k_{rel} *vs.* pH plots for dTMP/dGMP and dCMP/dTMP differ greatly from those for typical ozonization systems involving radical pathways. The complex variation of k_{rel} of dTMP/dGMP with pH may be ascribed to changes in the dissociation of bases (the dissociation constants of the base moiety, p*K*, are 2.9 and 9.7 for dGMP, and 10.0 for dTMP(9)). The results of stoichiometric determination, the effect of radical scavenger on k_{rel}, and the variation of k_{rel} with pH suggest that the ozonization reactions of dCMP, dTMP and dGMP in neutral solution do not involve a significant contribution of free radicals. On the other hand, the k_{rel} *vs.* pH plot for dAMP/dCMP and the appreciable effect of *tert*-butanol on k_{rel} suggest a contirubiton of radical attack on the 2′-deoxyribose moiety of dAMP.

Therefore, it is expected that, when ozone reacts with DNA, radical pathways are very minor processes, since the dGMP or dTMP moiety degrades much faster than the dAMP moiety, and consequently strand scission hardly occurs in the early stage of the reaction. Further study is being conducted with DNA to confirm this point, and the results will be reported elsewhere.

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