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Degradation of Nucleic Acids with Ozone. VI. Labilization of the Double-Helical Structure of Calf Thymus Deoxyribonucleic Acid[1)]

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The degradation of calf thymus deoxyribonucleic acid (DNA) (0.54 mg/ml) with ozone-containing oxygen gas (ozone content, 0.1 mg/l; flow rate, 70 ml/min) was examined. The degradation rates of the four nucleotides in native and heat-denatured DNAs were in the following order: dGMP $\simeq$ dTMP > dCMP $\simeq$ dAMP and dTMP > dGMP > dCMP $\simeq$ dAMP, respectively. In the case of heat-denatured DNA, the rapid degradation of the thymine moiety seems to be due to its relatively weak stacking ability, so that it is more exposed to attack. In intact DNA, the double-helical structure of DNA tends to protect the base moieties from attack by ozone. At the early stage of ozonization of native DNA, strand scission did not occur but the degradation of several guanine and/or thymine moieties was detected. As the ozonization proceeded, strand scissions of DNA and susceptibility to nuclease S_1 digestion were observed. Therefore a specific "ozone-denaturation" or "labilization" occurred, causing the double-helical structure to become increasingly loose due to the destruction of guanine and/or thymine moieties, and making the structure more accessible to nuclease S_1.

Keywords——ozone; ozonization; calf thymus DNA; DNA denaturation; nuclease P_1; nuclease S_1

Recently, there have been increasing interest in the use of ozone in drinking water and waste water treatment because of its superior oxidation and sterilization power. We are currently interested in improving the safety margin of safety cabinets and/or safety rooms, especially those used in recombinant deoxyribonucleic acid (DNA) experiments, by using ozone. Thus, studies on the mechanism of sterilization of bacteria and so forth, and on the degradation of nucleic acids are very important. Although there are several papers available on the mechanism of degradation (disinfection or inactivation) of viruses,[2)] bacteriophages,[3)] *E. coli*[4)] and lymphocytes[5)] by ozone, studies on the precise mode of degradation of DNA or ribonucleic acid (RNA) are considered to be essential for our purposes.

In the preceding paper,[1)] we reported that 2′-deoxyguanosine 5′-monophosphate and 2′-deoxythymidine 5′-monophosphate were degraded most rapidly by ozone at the base moieties by ionic reaction and not by ·OH radical reaction in neutral solution. Consequently, we suggested that, at the early stage of the ozonization of DNA, strand scission would hardly occur in neutral aqueous solution. In the present study, the degradation of calf thymus DNA with ozone was investigated to confirm this point and to clarify the mode of degradation of DNA. A preliminary communication was published in 1982.[6)]

Materials and Methods

Materials——Calf thymus DNA was obtained from Miles-Seravac Co. Nuclease P_1 and nuclease S_1 were

obtained from Yamasa Shōyu Co. and Seikagaku Kōgyō Co., respectively. All other chemicals used were of analytical grade.

Ozonization——The ozonization was carried out by bubbling ozone-containing oxygen gas (ozone content, 0.1 ± 0.01 mg/l; flow rate, 70 ml/min) into a 20 ml sample solution in a glass reactor equipped with a gas diffuser at 2 °C. Calf thymus DNA was dissolved in 40 mM phosphate buffer solution (pH 6.9, 0.15 M NaCl) at a concentration of 0.54 mg/ml. Heat-denatured DNA was prepared by heating DNA at 95 °C for 15 min followed by rapid cooling at 2 °C.

Analysis——Freshly ozonized samples were used for analysis. The amount of nucleotides generated after digestion with nuclease P_1 or nuclease S_1 was measured with a Hitachi model 635 high-performance liquid chromatograph (HPLC) at 264 nm[7] by using an anion-exchange column (pNH_2-column, Shimadzu Seisakusho, Ltd.) with 0.05 M KH_2PO_4–H_3PO_4 (pH 3.5) as the eluent at a flow rate of 1 ml/min. The column temperature was kept at 40 °C. All digestions mentioned below were performed in the presence of 20 mM phosphate.

Native DNA was completely digested at a weight ratio of DNA/nuclease $P_1 < 40$, at pH 5.5 and 50 °C for 60 min. Ozone-treated DNAs were digested under these conditions to estimate the amount of degradation in relation to the time of ozonization. The hydrolysis rate of ozone-treated DNA was also measured at a DNA/nuclease P_1 ratio of 54, at pH 5.5 and 37 °C. Under these conditions, the hydrolysis rates of native DNA and heat-denatured DNA by 1 mg of nuclease P_1 were 0.045 and 1.27 $\mu mol \cdot mg^{-1} \cdot min^{-1}$, respectively.

The conditions of digestion by nuclease S_1 were those presented in Sutton's paper.[8] A 200 μl aliquot of nuclease S_1 solution containing 3600 units was added to 200 μl of ozone-treated sample solution. The concentrations of Zn^{2+} and NaCl in the mixture were 0.06 and 105 mM, respectively. The mixture was kept at pH 4.5 and 45 °C for 90 min. Although the extent of digestion of intact heat-denatured calf thymus DNA was 70%, a good linear relationship was obtained between the amount of deoxynucleotides formed and the amount of heat-denatured DNA used in the concentration range of 0.010 to 0.108 mg.

The amount of malondialdehyde in a reaction mixture was measured by the thiobarbituric acid method[9] using a Hitachi 650-10S fluorescence spectrophotometer.

The melting temperature (T_m) of ozone-treated DNA was measured with a Hitachi 124 spectrophotometer.

Sucrose Density Gradient Centrifugation——A 5—20% (w/v) linear sucrose density gradient solution was prepared in 12.5 ml of 0.7 M NaCl–5 mM EDTA–0.3 N NaOH. A 500 μl aliquot of fresh ozone-treated DNA and/or intact DNA solution was added to 250 μl of 2.1 M NaCl–15 mM EDTA–0.9 N NaOH solution and the mixture was layered on top of a linear sucrose density gradient. The solution was centrifuged in an SW 40 rotor of a Beckman L2-65B ultracentrifuge for 15 h at 30000 rpm and 4 °C. After centrifugation, the gradient solution was fractionated by collecting 300 μl fractions and the ultraviolet (UV) absorbance of each fraction was measured at 260 nm after adding 400 μl of distilled water and 90 μl of 1 N HCl.

Results and Discussion

In this study, the degradation of calf thymus DNA was examined in the following ways: (1) determination of the amount of mononucleotides degraded by HPLC, (2) measurement of the strand scissions of DNA in terms of the amount of malondialdehyde generated, (3) assessment of the structural alteration of DNA by digesting the DNA with nuclease P_1 and nuclease S_1, and by measuring T_m.

The time courses of the degradation of native and heat-denatured DNA with ozone in terms of the mononucleotide contents are shown in Fig. 1. A control experiment was performed by bubbling oxygen gas alone through a sample solution for 120 min; no chemical change was detected. The degradation rates of the four nucleotides[10] in native and heat-denatured DNAs were in the order dGMP $\simeq$ dTMP > dCMP $\simeq$ dAMP and dTMP > dGMP > dCMP $\simeq$ dAMP, respectively. These orders were different from that of degradation of mononucleotides, dGMP > dTMP > dCMP > dAMP.[1] In particular, the rapid degradation of the thymine moiety in the heat-denatured DNA seems noteworthy (Fig. 1a). This may be due to the relatively weak stacking ability of the thymine moiety so that it tends to be located outside the ordered structure in the polynucleotide chain of heat-denatured DNA. In the double-helical native DNA, the thymine moiety, as well as other nucleotides, is protected from the attack of ozone.

Next, we examined whether strand scission can be caused by ozone. It is well known that malondialdehyde is generated immediately when DNA is subjected to radiation,[11] photodynamic action of 6-mercaptopurine[12] or the action of antitumor agents such as bleo-

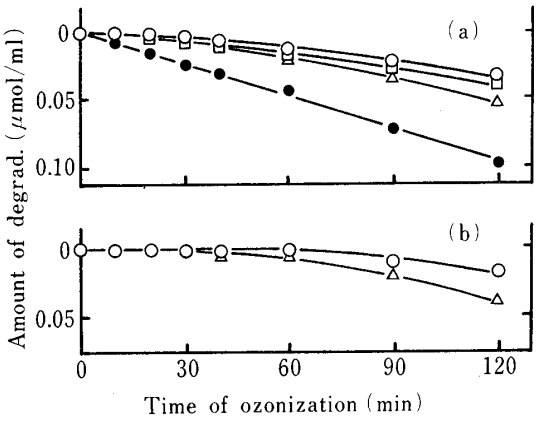

Fig. 1. Degradation of Mononucleotide Constituents of Calf Thymus DNA

a: heat-denatured DNA; —○—, dCMP; —□—, dAMP; —△—, dGMP; —●—, dTMP. b: native DNA; —○—, dCMP, dAMP; —△—, dGMP, dTMP.

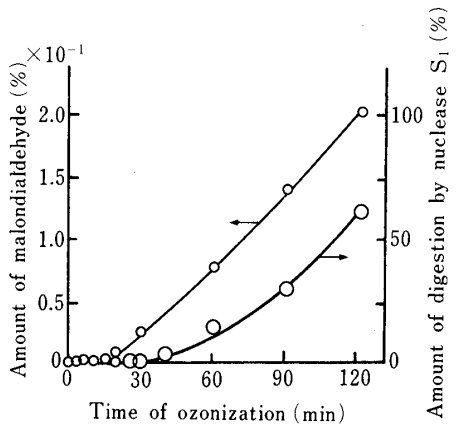

Fig. 2. Time Courses of Malondialdehyde Formation and Nuclease S_1 Digestion of Ozone-Treated Calf Thymus DNA

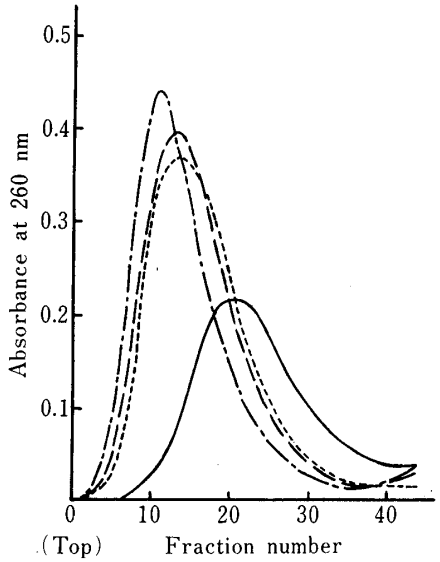

Fig. 3. Alkaline Sucrose Density Gradient Centrifugation Patterns of Ozone-Treated and Untreated Calf Thymus DNA

----, 0 min; ——, 3 min; —·—, 10 min/ozone-treated DNA. Neutral sucrose density gradient centrifugation patterns of untreated DNA and ozone-treated DNAs (10, 20 and 30 min) were all the same (shown by ——).

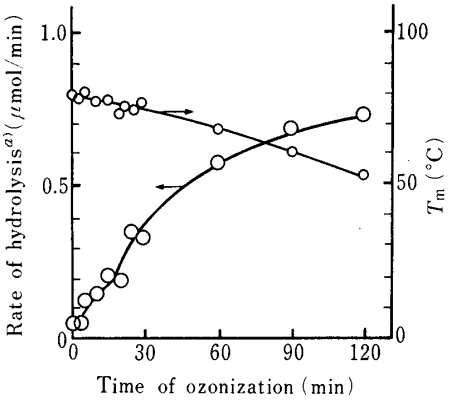

Fig. 4. Melting Temperature and Rate of Hydrolysis[a] of Ozone-Treated Calf Thymus DNA

a) The amount (μmol) of phosphodiester linkage hydrolyzed in 1 min by 1 mg of nuclease P_1 at 37 °C and pH 5.0. The rate of hydrolysis of denatured DNA was 1.27 μmol/min.

mycin.[13] However, in the case of ozonization, the formation of malondialdehyde was not observed within 20 min (Fig. 2). Since the formation of malondialdehyde is the result of the destruction of the 2-deoxyribose moiety of DNA by radicals such as ·OH,[11-13] it can be concluded that no strand scission occurred at the initial stage of ozonization of DNA. On the other hand, the patterns of alkaline sucrose density gradient centrifugation of ozone-treated DNAs were changed, as shown in Fig. 3, although the neutral sucrose density gradient centrifugation patterns were not shifted. This may be due to partial strand scission at the modified base moiety caused by alkaline treatment, as found in the treatment of tRNAs with

ozone.[14] Accordingly, the main initial reaction of ozone is concluded to be attack on the base moiety.[1,15]

A decrease of mononucleotides caused by ozone was not found until 30 min by HPLC. However, judging from the standard deviation of the analytical method using HPLC (*ca.* 1%) and from the results of alkaline sucrose density gradient centrifugation, several guanine and/or thymine moieties are considered to be degraded by ozone, and this would affect the double-helical structure, resulting in the formation of single-stranded regions. To confirm this, we measured the T_m values of ozone-treated native DNAs (Fig. 4) and digested the DNAs with nuclease S_1, which can digest single-stranded regions in the DNA (Fig. 2). In addition, the rates of hydrolysis of ozone-treated DNAs by nuclease P_1 were measured, since it is well known that the rate of hydrolysis of heat-denatured DNA by nuclease P_1 is about two hundred times that of native DNA[16] (Fig. 4). Though the hydrolysis rates by nuclease P_1 increased and the T_ms decreased with the time of ozonization, no nuclease S_1 digestion was observed within 30 min. This suggests that while the hydrogen bonds in the degraded guanine and/or thymine moieties were weakened, the overall double-helical structure of DNA remained intact, during this period.

After 30 min of ozonization, degradation of the base moieties and several strand scissions of DNA were observed. At 120 min, the amount of total base damage and strand scissions reached 7 and 0.2%, respectively, while the amount of single-stranded regions reached 60%. In contrast to the case of denatured DNA (Fig. 1a), the degradation rate of the thymine moiety was about the same as that of the guanine moiety in ozone-treated native DNA. Thus, it may be considered that specific "ozone-denaturation" or "relaxation" did occur, leading to loosening of the double helical-structure due to destruction of the guanine and/or thymine moieties, so that nuclease S_1 became able to attack the structure.

As mentioned above, ozonization of calf thymus DNA has provided preliminary results on the mode of ozone-degradation of DNA. Further studies on the precise mode of degradation are being carried out on several plasmid DNAs for which total gene maps are clearly defined, and the results will be reported elsewhere.

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