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Effects of Acid and Temperature on the Activity of Bleomycin

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The ability of bleomycin to decrease the melting temperature (T_m) of deoxyribonucleic acid (DNA) in the presence of 1,2-benzenediol (catechol) was enhanced by previous storage of bleomycin in a solution of pH 3.0 for 3 h. Although this enhancement was also caused by adding water which was repeatedly (5 times) adjusted to pH 3.0 and then back to 7.4 without bleomycin, it was lost by further storage for 24 h at 37 °C after neutralization to pH 7.4. These findings suggest that the activity of bleomycin may be indirectly enhanced by acid. The present result that bleomycin could effectively break the DNA strand at 60 °C in comparison with other temperatures tested shows that the drug can preferentially interact with the loose helix of DNA.

Keywords——bleomycin; catechol; 2-mercaptoethanol; pH effect; temperature dependence; DNA melting temperature; DNA strand breakage

The *in vitro* activities of bleomycin for breaking the strand of deoxyribonucleic acid (DNA), releasing free bases from DNA and decreasing the melting temperature (T_m) of DNA were enhanced by ultraviolet (UV) irradiation.[1–3] Recently, it has been reported that the 2,4′-bithiazole rings in the bleomycin molecule phototransformed to 4,4′-bithiazole and that the breakage activity and the preferential recognition of the nucleotide sequence of the phototransformed bleomycin did not differ from those of the original bleomycin.[4] It has been suggested that the ability of bleomycin to decrease T_m of DNA might be enhanced in some other manner besides UV irradiation.[5] In this study, as the activity of bleomycin is sensitively influenced by the conditions, the effect of pH during storage of bleomycin on the ability to decrease T_m and the effect of temperature on the activity for breaking the DNA strand were investigated.

Bleomycin can decrease T_m of DNA in the presence of 1,2-benzenediol (catechol).[5] Figure 1 shows the enhancement of the ability of bleomycin to decrease T_m of DNA by previous storage for 3 h at pH 3.0. Bleomycin stored at pH 3.0 (40 μM) decreased T_m to 57.5 °C

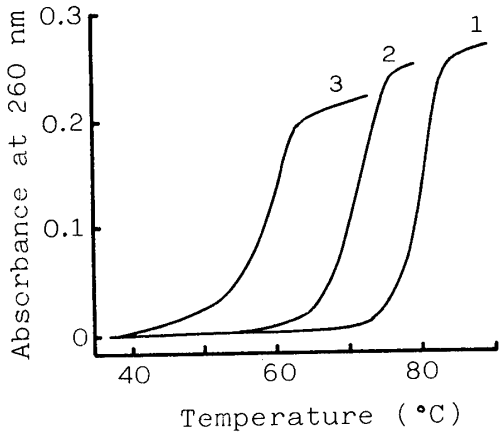

Fig. 1. Thermal Melting Curves of DNA Treated with 40 μM Bleomycin, Stored or Not in a Solution of pH 3.0 for 3 h, in the Presence of 1 mM Catechol Measured by Using the Optical Density Method

1, DNA+catechol; 2, DNA+catechol+original bleomycin; 3, DNA+catechol+bleomycin stored at pH 3.0.

TABLE I. Effects of "Acid-Alkali Treatment" of 40 μM Bleomycin on T_m of DNA in the Presence of 1 mM Catechol Measured by Using the Optical Density Method

Treatment of bleomycin	T_m of DNA (°C)
Bleomycin free	80.0
Original	70.5
"Acid-alkali treatment"	56.5
Incubation at pH 3.0 after "acid-alkali treatment"	56.5
Incubation at pH 7.4 after "acid-alkali treatment"	57.5

Bleomycin solution (48 μM) was treated by "acid-alkali treatment" as described in Experimental or incubated at each pH value for 24 h after "acid-alkali treatment." Each reaction mixture contained 50 μg/ml salmon sperm DNA, 1 mM catechol and 40 μM bleomycin mentioned above in 50 mM Tris–HCl and 1 mM KCl buffer of pH 7.4, and was incubated at 37 °C for 2 h.

TABLE II. Effects of "Acid-Alkali Treatment" of Water on T_m of DNA Treated with 40 μM Bleomycin in the Presence of 1 mM Catechol Measured by Using the Optical Density Method

Treatment of water	T_m of DNA (°C)
Notreatment	70.5
"Acid-alkali treatment"	60.5
Incubation at pH 7.4 after "acid-alkali treatment"	70.0

Each reaction mixture was prepared as described in the footnote to Table I. Water subjected to "acid-alkali treatment" or incubated for 24 h at pH 7.4 after "acid-alkali treatment" was added to make 1/3 of the total volume of the reaction mixture.

in the presence of 1 mM catechol in 50 mM tris(hydroxymethyl-aminomethane (Tris)–HCl and 1 mM KCl buffer of pH 7.4, whereas T_m was 70.5 °C in the case of the original bleomycin. As the same volume of NaCl solution prepared by neutralization of 2 N HCl by adding 2 N NaOH was added to the reaction mixture containing the original bleomycin, it seems that the enhancement of the ability of bleomycin to decrease T_m of DNA caused by storage in a solution of pH 3.0 is not attributable to the addition of a trace amount of ferrous ions present in the HCl and/or NaOH.

Table I shows the effect of "acid-alkali treatment" on the ability of bleomycin to decrease T_m of DNA. As well as the effect of storage at pH 3.0, the activity of bleomycin was enhanced by "acid-alkali treatment"; *i.e.*, 40 μM bleomycin decreased T_m to 56.5 °C in the presence of 1 mM catechol in 50 mM Tris–HCl and 1 mM KCl buffer of pH 7.4. This effect did not change with incubation for 24 h at 37 °C in a solution of pH 3.0 or 7.4.

Moreover, such enhancement of the activity of bleomycin was also caused by the addition of water treated by "acid-alkali treatment" to the reaction mixture containing 40 μM original bleomycin and 1 mM catechol in 50 mM Tris–HCl and 1 mM KCl buffer of pH 7.4 as summarized in Table II. Its T_m was 60.5 °C. However, when the water was neutralized to pH 7.4 after "acid-alkali treatment" and incubated for 24 h at 37 °C, the enhancement was lost; that is, T_m became 70.0 °C corresponding to T_m of DNA reacted with the original bleomycin. Therefore, it is suggested that the effect of "acid-alkali treatment" on the activity of bleomycin may be caused indirectly rather than by a direct effect on the bleomycin molecule.

It has been shown that oxygen and ferrous ions play an important role in the activation of bleomycin and that the active Fe(II)·bleomycin is formed from Fe(II)·bleomycin complex in the presence of oxygen and electron(s).[6-8] Therefore, it seems that the enhancement of the activity of bleomycin by "acid-alkali treatment" may relate to oxygen or ferrous ions.

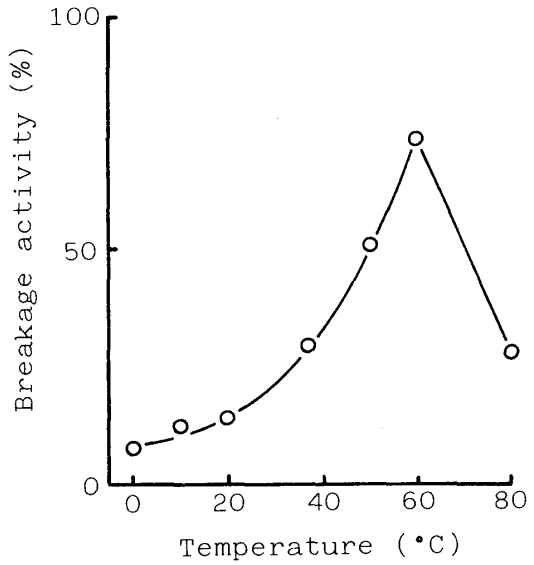

Fig. 2. Plot of the Breakage Activity of the DNA Strand *versus* Reaction Temperature of 40 μM Bleomycin in the Presence of 1 mM 2-Mercaptoethanol at pH 8.1

Although the details of this phenomenon are unclear, the present results show that another means besides UV irradiation for increasing the activity of bleomycin is storage of the drug in an acidic solution.

The effect of temperature on the breakage of the DNA strand caused by bleomycin is shown in Fig. 2. In the presence of 1 mM 2-hydroxy-1-ethanethiol (2-mercaptoethanol), the DNA strand breakage activity of 40 μM bleomycin in 50 mM Tris–HCl and 1 mM KCl buffer of pH 8.1 was about 10% in the temperature range from 0 to 20 °C and increased steeply in the range from 37 to 60 °C. The optimal temperature was 60 °C (breakage activity of 75%). When the temperature was raised to 80 °C, the breakage activity became 25%. The present finding differs from the report that the optimal temperature for breaking the single strand of simian virus 40 DNA was 20 °C at pH 9.1.[9] It is considered that the difference is due to the different conformations of DNA, *i.e.*, linear DNA and closed circular DNA. Interestingly, the findings on the thermal melting curve measured by using the optical density and viscosity methods showed that the double helix of DNA became unstable near 65 °C and was disrupted at 95 °C and that T_m was 76 °C in 50 mM Tris–HCl and 1 mM KCl buffer of pH 8.1 (data not shown). It is suggested that bleomycin can attack a loose form of the double-helical DNA more effectively than a rigid one.

In conclusion, the *in vitro* activity of bleomycin is very sensitive to the conditions of storage and to the conformation of DNA as the substrate; a loose helix of DNA is more susceptible to bleomycin.

Experimental

Materials——Bleomycin was obtained from Nippon Kayaku Co. Highly polymerized salmon sperm DNA and 3,8-diamino-5-ethyl-6-phenylphenanthridinium bromide (ethidium bromide) were obtained from Sigma Co. and catechol, 2-mercaptoethanol and other reagents were obtained from Wako Pure Chemical Industries Co. So-called reversible DNA, salmon sperm DNA treated with HNO_2, was prepared by the procedure of Geiduschek.[10] Reversible DNA having 90% reversibility was used to examine the strand breakage of DNA caused by bleomycin.

Preparation of Bleomycin Solution in Water Treated with 2N HCl and 2N NaOH——A solution of 48 μM bleomycin in 50 mM Tris–HCl buffer of pH 3.0 was stored for 3 h at 37 °C and neutralized to pH 7.4 by adding 2 N NaOH. Another method of treatment of bleomycin solution or water involved the steep variation of pH. That is, the pH value of a solution or water was adjusted to pH 3.0 by adding 2 N HCl and immediately readjusted to pH 7.4 by adding 2 N NaOH. This treatment was repeated 5 times. In this study, this procedure is called "acid-alkali treatment." The above-mentioned solution of pH 7.4 was added to the reaction mixture for obtaining the thermal melting curve

of DNA. The same volume of NaCl solution prepared by neutralization of 2 N HCl by adding 2 N NaOH was added to the original bleomycin solution. No difference of UV spectra between the two bleomycin solutions was apparent. Correction of the bleomycin concentration was carried out by measuring the absorbance at 293 nm with a Hitachi EPS-3T spectrophotometer.

Examination of Thermal Melting Curve of DNA Treated with Bleomycin Measured by Using the Optical Density Method——The following reaction mixture was prepared; 50 μg/ml salmon sperm DNA ($A_{260}=0.6$), 40 μM bleomycin subjected to "acid-alkali treatment" or not (in the preparation of the solution treated with "acid-alkali treatment," the bleomycin concentration fell from an initial value of 48 to 40 μM) and 1 mM catechol in 50 mM Tris–HCl and 1 mM KCl buffer of pH 7.4. Water, subjected to "acid-alkali treatment" was added to make 1/3 of the total volume of the reaction mixture. Each reaction mixture was incubated for 2 h at 37 °C and the absorbance was measured at 260 nm with a Hitachi EPS-3T spectrophotometer in a cuvette (10 mm light path) with a stopper to obtain the thermal melting curve in the temperature range from 37 to 95 °C; the temperature was raised by one degree every 2 min. the parameter, T_m, characterizing the melting transition corresponds to the temperature at which the rise in A_{260} was half complete.

Examination of DNA Strand Breakage Caused by Bleomycin——The reaction mixture containing reversible DNA at the concentration of $A_{260}=1.0$, 40 μM bleomycin and 1 mM 2-mercaptoethanol in 50 mM Tris–HCl and 1 mM KCl buffer of pH 8.1 was prepared and incubated for 3.5 h at various temperatures, *e.g.*, 0, 10, 20, 37, 50, 60 and 80 °C. The breakage activity of the DNA strand in each reaction mixture was assayed by the reported method.[11] That is, each reaction mixture was denatured in boiling water for 3 min and quenched immediately in ice. Finally, 500 μg/ml ethidium bromide, dissolved in a solution of 20 mM K_2HPO_4 and 200 μM ethylenediaminetetraacetic acid adjusted to pH 11.5, was added to each reaction mixture. The fluorescence intensity of the final solution of pH 11.3 was measured at 590 nm (excited at 525 nm) with a Hitachi MPF-3 fluorescence spectrometer. The breakage activity of the DNA strand (%) was estimated by using the following equation;

$$\text{breakage activity (\%)} = 100 - \frac{\text{fluorescence intensity of reaction mixture treated with bleomycin}}{\text{fluorescence intensity of reaction mixture not treated with bleomycin}} \times 100 .$$

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