REACTION OF BLEOMYCIN WITH DNA

STRAND SCISSION OF DNA IN THE ABSENCE OF SULFHYDRYL OR PEROXIDE COMPOUNDS

Isao Shirakawa, Masao Azegami,

Sin-ichi Ishii and Hamao Umezawa*

Faculty of Pharmaceutical Sciences, Hokkaido University,
Sapporo, Japan
* Institute of Microbial Chemistry, Shinagawa-ku, Tokyo, Japan

The effect of bleomycin on the infectivity and the molecular integrity of bacteriophage ϕX 174 DNA was examined by means of the spheroplast assay and sucrose density gradient centrifugation.

Bleomycins caused degradation of the DNA, both in the single and double stranded forms even in the absence of 2-mercaptoethanol or hydrogen peroxide, although the reaction was accelerated by the addition of either of the compounds. All the bleomycin compounds examined, A_2, A_5 and B_2, were effective for the reaction but copper-chelated A_2 was not. The reaction was inhibited by the addition of EDTA*.

Bleomycins are antibiotics which are produced by *Streptomyces verticillus* and have antitumour and antibacterial activities[1,2,3].

It has been reported that bleomycins inhibit DNA synthesis rather than protein or RNA synthesis in *Escherichia coli*, Ehrlich carcinoma and HeLa cells[4]. They cause strand scissions in DNA of growing *E. coli*, and HeLa cells[5]. They bind to DNA and decrease the melting temperature (Tm) and the molecular size in the presence of sulfhydryl compounds or hydrogen peroxide, *in vitro*[6,7,8,9]. It has been suggested that sulfhydryl compounds or hydrogen peroxide may take part in the *in vivo* action of bleomycin[5,8].

In the study of effects of bleomycin on the growth of bacteriophage ϕX174, which has a single-stranded circular DNA (phage DNA) and also produces double-stranded circular DNA called replicative form (RF)** in the host cell during the development, the degradation of the single-stranded phage DNA and the double-stranded RF DNA was observed *in vitro* in the absence of sulfhydryl compounds or hydrogen peroxide. The results are presented in this paper.

Materials and Methods

The following bleomycins were used: mixture (lot. F-7, A_2; 69.0 %, B_2; 28.8 %, A_2'; 1.5 %, B_1; 1.5 %, A_5; 0.9 %, A_1; 0.5 %). A_2 (lot. F1-8), A_5 (lot. 3, spd), A_2(Cu) (lot. F1-8, copper-chelated), B_2 (lot. 32). All bleomycins were kindly offered by Nippon Kayaku

---

* EDTA : ethylenediaminetetraacetic acid.
** RF : RF-I (both strands are closed) and RF-II (one or both strands are nicked).

Co., Tokyo.   (Methyl-³H) thymidine (specific activity 15.5 c/m mole) was purchased from Schwarz Bioresearch, Inc.   Bacterial and phage strains were given to us by Dr. R. L. SINSHEIMER.

Zone Sedimentation and Radioactivity Measurement

DNA or its reaction mixture (in 0.2~0.6 ml) was layered on a 5~20 % sucrose density gradient in 0.05 M tris – 0.5 M sodium chloride – 1 mM EDTA (pH 7.6, Tris – saline – EDTA buffer) and centrifuged at 4°C in an SW 27 rotor of a Beckman L2-65B centrifuge at 25,000 r.p.m. for 16 hours.   Fractions were collected from the bottom of tubes.   The DNA in each fraction was precipitated with cold 5 % (final concentration) trichloroacetic acid (TCA) after adding 100 μg of herring sperm DNA as a carrier.   Precipitates containing ³²P or ³H-DNA were collected on glass fibre paper (Whatman GF/C), dried and counted in an Aloka scintillation counter (toluene base scitillator).

Bioassay

The biological activity of the phage DNA was measured by the spheroplast assay as described by GUTHRIE et al.[10]

Preparation of Phage DNA

³²P-Labeled ϕX174 am3 (lysis defective mutant) was prepared essentially according to the procedure of SINSHEIMER et al.[11]   ³²P-Labeled ϕX174 am3 DNA was prepared by the method of GUTHRIE et al.[10]   The phage DNA was purified by sucrose density gradient centrifugation, if necessary.   Fractions containing ³²P-phage DNA were pooled and the DNA was precipitated with 2.5 volumes of ethanol after the addition of tRNA (10 μg/ml) or the cold phage DNA (80 μg/ml) as a carrier.   Precipitates were dissolved in 0.05 M tris buffer (pH 7.6 or 7.4).

Preparation of ³H-Labeled RF DNA

E. coli HF 4704 (hcr⁻, thymine requiring) was grown in TPG-2A medium[11] containing 5 μg/ml of thymine to about 1×10^8 cells/ml at 37°C with aeration.   The cells were incubated after the addition of 30 μg/ml of mitomycin C for 10 minutes without aeration, collected by centrifugation and resuspended in the fresh medium containing 2 μg/ml of thymine. The cells were infected with ϕX174 am3 and at the same time 0.02 mc/ml of ³H-thymidine and 30 μg/ml of chloramphenicol were added.   The infected cells were incubated further for 3 hours under vigorous aeration, then collected, washed and resuspended in 0.05 M tris buffer (pH 8.1).   The cells were lysed by lysozyme-EDTA treatment[10], followed by freezing and thawing.   The lysate was shaken with an equal volume of phenol saturated with 0.05 M tris buffer (pH 8.1) and the aqueous phase was recovered after separation by centrifugation.   Remaining phenol in the aqueous phase was removed by the extraction with ether.   After adding 0.1 volume of 3 M sodium acetate (pH 5.5), 2.5 volumes of isopropanol and 10 μg/ml of yeast tRNA, the solution was allowed to stand in a freezer overnight.   The precipitate was collected by centrifugation.

Results

Effects of Bleomycin A₂ on Infectivity of Single-stranded
Phage DNA to Spheroplast

If ϕX174 phage DNA, single-stranded and closed, is opened by one strand scission or suffers chemical modification in some base residues by the action of hydroxylamine or other reagents, its infectivity to E. coli spheroplast is known to be lost.   Therefore this spheroplast assay system gives a very sensitive detection method for DNA alteration.   The effect of bleomycin A₂ on the infectivity of the single-stranded phage DNA to the spheroplast is shown in Fig. 1.   The inactivation of phage DNA by 1 μg/ml of bleomycin A₂ was observed in the presence of neither 2-mercaptoethanol nor hydrogen peroxide.

Fig. 1. Inactivation of phage DNA by bleomycin A_2 as revealed
by spheroplast assay.

The phage DNA (about 1×10^{-3} μg/ml) was incubated with bleomycin A_2 in 0.05 M tris buffer
(pH 7.6) at 37℃. A portion of reaction mixture at the time indicated was transferred and
diluted into ice-cold 0.05 M tris buffer (pH 8.1) containing yeast tRNA (2 μg/ml). Dilution was
performed until the bleomycin present caused no effect on the spheroplast with respect to the
infectivity of the phage DNA. The biological activity of the treated phage DNA was measured
in the spheroplast assay as described by GUTHRIE et al.[10]

    a. untreated   b. bleomycin A_2 (5 μg/ml)   c. bleomycin A_2 (1 μg/ml)

    To the reaction mixture (c), a half volume of 10 mM EDTA was
added after 30 minutes.
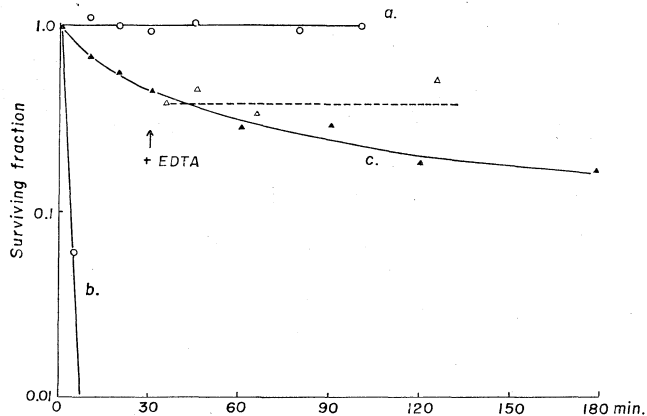
Degradation of Single-stranded or Double-stranded DNA by Bleomycin in the Absence of 2-Mercaptoethanol or Hydrogen Peroxide

Effects of three different types of bleomycin, A_2, A_5 and B_2, on the phage DNA
were examined by means of sucrose density gradient centrifugation. As shown in

Fig. 2. Effects of bleomycin A_2 and A_2(Cu) and the inhibition
of the bleomycin activity by EDTA.

The phage DNA (1×10^{-3} μg/ml) was incubated with bleomycin in 0.05 M tris buffer (pH 7.6)
at 37℃ for 90 minutes. The reaction mixture was layered on a $5 \sim 20$ % sucrose density gradient
in Tris-saline-EDTA buffer and centrifuged as described in materials and methods. Each
fraction was collected in a planchet, dried and counted in an Aloka GM counter.

    A. a. untreated   b. bleomycin A_2(Cu) (5 μg/ml)

    B. a. untreated   b. bleomycin A_2 (5 μg/ml)

    c. bleomycin A_2 (5 μg/ml)+EDTA (2.2 mM)
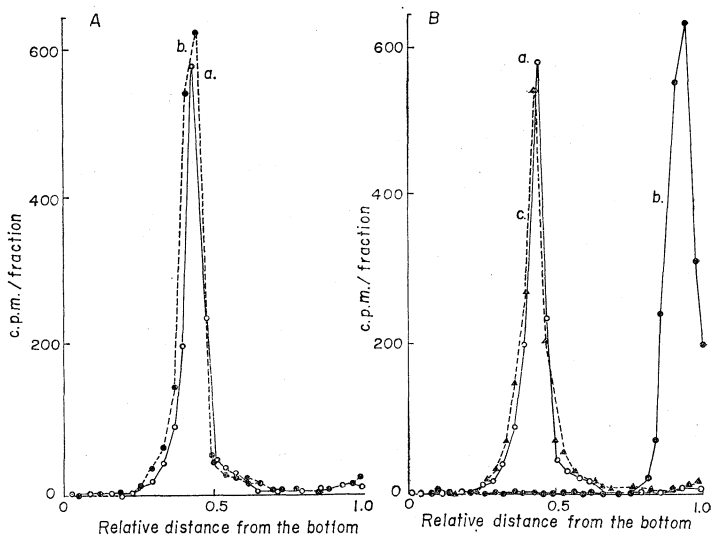
Fig. 3. Effects of various bleomycins on the sedimentation behaviour of the phage DNA.

The phage DNA (about 0.7 μg/ml) was incubated with bleomycin in 0.05 M tris buffer (pH 7.4) at 37℃ for 120 minutes. The reaction mixture was centrifuged and counted as described in the legend of Fig. 2.

    a. untreated   b. bleomycin B_2 (50 μg/ml)
    c. bleomycin A_5 (50 μg/ml)

Fig. 4. Effect of bleomycin on double-stranded RF DNA.

The RF DNA (about 3×10^{-2} μg/ml) was incubated with bleomycin in 0.05 M tris buffer (pH 7.6) at 37℃ for 90 minutes. The DNA in the reaction mixture was precipitated in the cold with the addition of 0.1 volume of tRNA (1 mg/ml), 0.1 volume of 3 M sodium acetate (pH 5.5) and 2 volumes of ethanol and then redissolved in 0.05 M tris buffer (pH 7.6). The dissolved DNA was centrifuged and counted as described in materials and methods.

    a. untreated
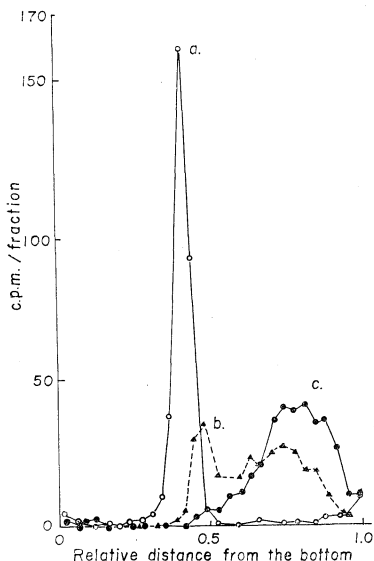   b. bleomycin mixture (50 μg/ml)

Fig. 2B and 3, the phage DNA treated with bleomycins A_2, A_5 and B_2 showed a decrease in the sedimentation velocity. The double-stranded RF DNA was also degraded by bleomycin as indicated in Fig. 4, where a specimen of bleomycin mixture was used. Fig. 5 presents the result of another experiment to reveal the degradation of phage DNA by bleomycin. The amount (as radioactivity) of TCA-insoluble portion of the DNA was found to decrease during the treatment with bleomycin mixture.

Action of Bleomycin in Cooperation with 2–Mercaptoethanol or Hydrogen Peroxide

The amount of TCA-insoluble portion from the DNA treated with bleomycin decreased more rapidly in the presence of 2–mercaptoethanol than in the absence of that (Fig. 5). The effect of hydrogen peroxide on the DNA degradation by bleomycin is illustrated in Fig. 6. The sedimentation pattern of phage DNA treated with 3.3 μg/ml of bleomycin A_2, at which concentration only a little change was caused in the absence of hydrogen peroxide, showed a remarkable degradation upon the further addition of
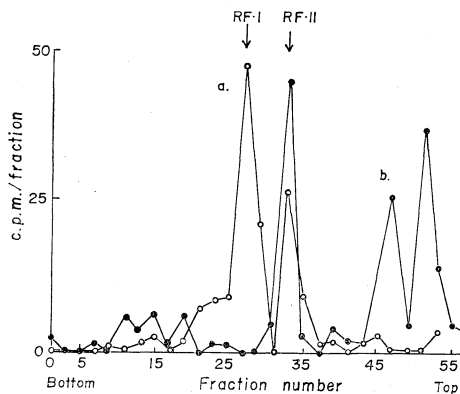
Fig. 5. Effect of 2-mercaptoethanol on bleomycin activity.

The phage DNA (about 1×10^{-2} μg/ml) was incubated with bleomycin mixture in 0.05 M tris buffer (pH 7.6) at 37℃. A portion of the sample at the time indicated was added with carrier DNA to make a final concentration of 100 μg/ml and with cold TCA at a final concentration of 5 %. The precipitates were collected on glass fibre paper, dried and counted in a scintillation counter.

    a. untreated
    b. bleomycin mixture (3.4 mg/ml)
    c. bleomycin mixture (3.4 mg/ml)
       +2-mercaptoethanol (1 mM)
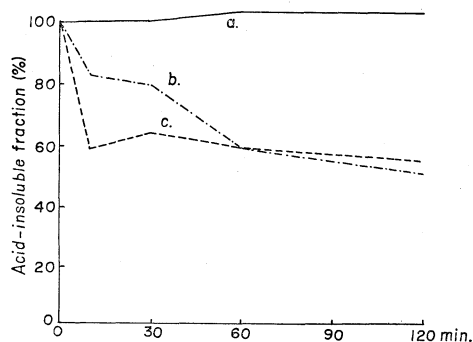
0.03 mM of hydrogen peroxide. The pattern of
DNA treated with hydrogen peroxide only was
the same as that of untreated DNA.

Ineffectiveness of Copper-chelated
Bleomycin and Inhibition by EDTA
of the Bleomycin Action

The copper-chelated bleomycin has been
reported not to bring about the observable
strand scission of DNA even in the presence
of 2-mercaptoethanol. It is also known that
the strand scission of DNA with bleomycin in
the presence of 2-mercaptoethanol is inhibited
by an addition of EDTA[4,5,6,7,8]. We examined
whether these effects held also for the bleo-
mycin action on the phage DNA without 2-
mercaptoethanol. As shown in Fig. 2 A,
copper-chelated bleomycin A_2 did not cause
the observable degradation of DNA in the
absence of 2-mercaptoethanol, while bleomycin
A_2 freed from copper by the dithizon treat-
ment recovered the activity (not indicated here).
The activity of bleomycin A_2 was inhibited
rapidly by the addition of EDTA in the
absence of 2-mercaptoethanol as shown in the
sedimentation (Fig. 2B) or in the spheroplast
assay experiment (Fig. 1). These results are consistent with those previously reported.

Fig. 6. Effect of hydrogen peroxide
on bleomycin activity.

The phage DNA (about 1×10^{-3} μg/ml) was in-
cubated with bleomycin A_2 in 0.05 M tris buffer
(pH 7.6) at 37℃ for 70 minutes. The reaction
mixture was centrifuged and counted as des-
cribed in the legend of Fig. 2.
  a. untreated
  b. bleomycin A_2 (3.3 μg/ml)
  c. bleomycin A_2 (3.3 μg/ml)
      + hydrogen peroxide (0.03 mM)
 d. hydrogen peroxide (0.03 mM)

Discussion

The biological activity of bleomycins has been discussed in connection with DNA
degradation[6,8]. ^{3}H-Labeled bleomycin has been reported to bind to DNA and to cause
strand scission and degradation of DNA in the presence of 2-mercaptoethanol or hydrogen
peroxide but not in their absence *in vitro* even though it binds to DNA. These conclusions
were deduced from the observed change in melting temperature or sedimentation patterns.
In our studies it was found that, even in the absence of 2-mercaptoethanol or hydrogen
peroxide, bleomycin(s) inactivated the biological activity of phage DNA and moreover
degraded both the phage DNA and the double-stranded RF DNA. 2-Mercaptoethanol or
hydrogen peroxide only enhanced the degradation of DNA by bleomycin. Consistent with
the results of previous authors, the chelation of copper to bleomycin or the addition of
EDTA to the reaction mixture inhibited the *in vitro* activity of bleomycin in the present
system.

ϕX 174 Phage DNA and RF DNA used in our experiments possess several advantages
for the detection of alteration in their molecular integrity. For example, the formation
of only a single nick in the DNA strand may be easily revealed, because the circular
closed native strand and the resulted linear open strand can be separated from each other
by zone sedimentation. Moreover the spheroplast assay can afford a very sensitive detec-
tion method for DNA alteration as mentioned above[12]. Presumably, the sensitivity of our
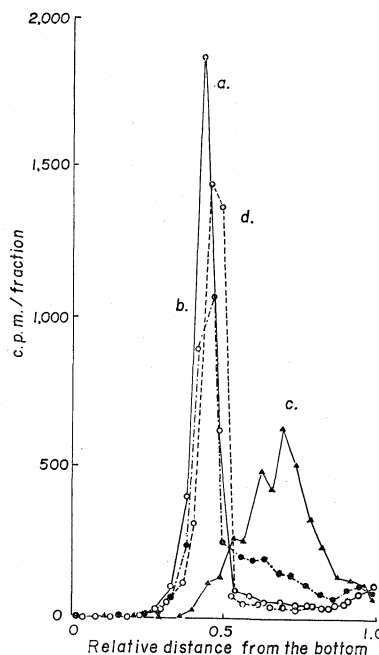
system detects strand scissions not detected previously.

Although the biological activity of bleomycin *in vivo* might be considered in connection with sulfhydryl or peroxide compounds, their stimulation of DNA degradation by bleomycin seems not to be indispensable, at least *in vitro*.

<div align="center">References</div>

1) UMEZAWA, H.; K. MAEDA, T. TAKEUCHI & Y. OKAMI : New antibiotics, bleomycin A and B. J. Antibiotics, Ser. A 19 : 200~209, 1966

2) UMEZAWA, H.; Y. SUHARA, T. TAKITA & K. MAEDA : Purification of bleomycins. J. Antibiotics, Ser. A 19 : 210~215, 1966

3) ISHIZUKA, M.; H. TAKAYAMA, T. TAKEUCHI & H. UMEZAWA : Activity and toxicity of bleomycin. J. Antibiotics, Ser. A 20 : 15~24, 1967

4) SUZUKI, H.; K. NAGAI, H. YAMAKI, N. TANAKA & H. UMEZAWA : Mechanism of action of bleomycins. Studies with the growing culture of bacterial and tumor cells. J. Antibiotics 21 : 379~386, 1968

5) SUZUKI, H.; K. NAGAI, H. YAMAKI, N. TANAKA & H. UMEZAWA : On the mechanism of action of bleomycin : Scission of DNA strands *in vitro* and *in vivo*. J. Antibiotics 22 : 446~448, 1969

6) NAGAI, K.; H. YAMAKI, H. SUZUKI, N. TANAKA & H. UMEZAWA : The combined effects of bleomycin and sulfhydryl compounds on the thermal denaturation of DNA. Biochim. Biophys. Acta 179 : 165~171, 1969

7) NAGAI, K.; H. SUZUKI, N. TANAKA & H. UMEZAWA : Decrease of melting temperature and single strand scission of DNA by bleomycin in the presence of 2-mercaptoethanol. J. Antibiotics 22 : 569~573, 1969

8) NAGAI, K.; H. SUZUKI, N. TANAKA & H. UMEZAWA : Decrease of melting temperature and single strand scission of DNA by bleomycin in the presence of hydrogen peroxide. J. Antibiotics 22 : 624~628, 1969

9) SUZUKI, H.; K. NAGAI, E. AKUTSU, H. YAMAKI, N. TANAKA & H. UMEZAWA : On the mechanism of action of bleomycin. Strand scission of DNA caused by bleomycin and its binding to DNA *in vitro*. J. Antibiotics 23 : 473~480, 1970

10) GUTHRIE, G. D. & R. L. SINSHEIMER : Observations on the infection of bacterial protoplasts with the deoxyribonucleic acid of bacteriophage φX174. Biochim. Biophys. Acta 72 : 290~297, 1963

11) LINDQVIST, B. H. & R. L. SINSHEIMER : Process of infection with bacteriophage φX174. XIV. Studies on macromolecular synthesis during infection with a lysis-defective mutant. J. Mol. Biol. 28 : 87~94, 1967

12) BURTON, A. & R. L. SINSHEIMER : The process of infection with bacteriophage φX174. VII. Ultracentrifugal analysis of the replicative form. J. Mol. Biol. 14 : 327~347, 1965