THE EFFECT OF BLEOMYCIN ON SV40 DNA: CHARACTERISTICS OF BLEOMYCIN ACTION WHICH PRODUCES A SINGLE SCISSION IN A SUPERHELICAL FORM OF SV40 DNA

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The effect of bleomycin on the molecular integrity of SV40 DNA was determined under various conditions by taking advantage of the fact that a single scission produced in such a superhelical form of DNA could be clearly manifested by analysis of samples with centrifugation in alkaline sucrose gradients. Conditions were found where the reaction velocity, the rate of disappearance of the characteristic form of DNA, depended on the concentration of bleomycin as well as on the reaction time. In contrast to general enzymatic reactions, bleomycin activity was greater at 0°C than at 37°C. The optimum pH was 9.1 while no activity was observed below pH 6 or above pH 13. Additional DNA, irrespective of its source, inhibited the reaction, while RNA had no effect. Cu++ ion at 1 mM almost completely inhibited the reaction while 2-mercaptoethanol at 1 mM stimulated the reaction by about 20-fold. No sulfhydryl compound was included in our standard assay system. EDTA at 10 mM showed no effect.

Bleomycin treatment produces a scission of DNA strands both *in vivo* and *in vitro*[1~6]. In this action, bleomycin neither requires the native conformation[7] nor a particular base composition of DNA[18]. The fragmentation of DNA by bleomycin, which can be enhanced by addition of reducing or oxidizing agents[8,9], is accompanied by release of free bases[10] and aldehyde functions[8]. However, the detailed reaction sequence remains to be elucidated. To characterize bleomycin action, it seemed of primary importance to find reaction conditions where the rate of DNA fragmentation could conveniently be followed as a function of time and drug concentration. Use of SV40 DNA as substrate is of major advantage since its native structure is double-stranded, covalently closed-circular and superhelical. It sediments with 53S at an alkaline pH, and if a single scission is produced on either strand of the native form, the products sediment with 18S (single-stranded, closed-circular DNA) and 16S (single-stranded, linear DNA) under the same conditions[11]. Under restricted conditions, 16S and 18S DNA were found to be the major products of bleomycin action because amounts of slower sedimenting DNA fragments or acid soluble material were negligible. Since bleomycin can act efficiently on DNA even at 0°C, as will be described below, a critical aspect of our assay system was the use of an extremely alkaline pH to terminate the reaction. With this assay, reaction velocities as a function of bleomycin concentration were determined as was the effect of temperature, pH and various additives on the bleomycin activity. In the present paper, these results are presented and the probable mechanism of bleomycin action is discussed.

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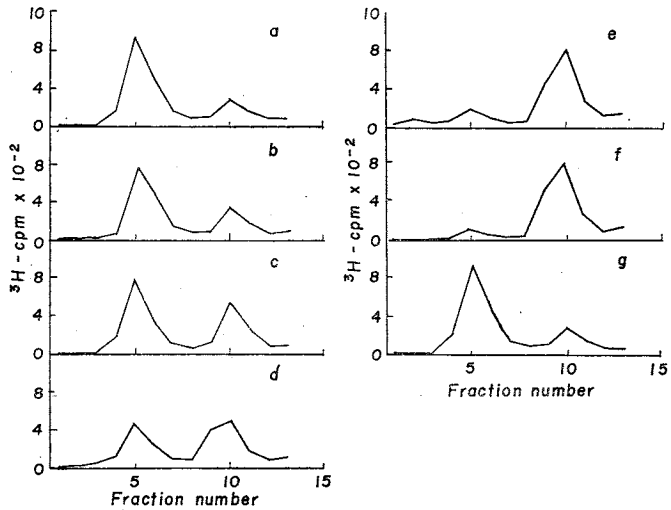
Materials and Methods

Bleomycin B_2 (lot 32, copper-free), supplied by Nihon Kayaku Co., Ltd., Tokyo, was dissolved in 0.1 M Tris-HCl, pH 7.5 (adjusted at 20°C), or in other appropriate buffered solutions (see legends to Figs. 3 and 4), at a concentration of 160 μg/ml and refrigerated until use. An inactive derivative of bleomycin B_2, prepared by treating bleomycin B_2 with an enzyme from mouse liver was obtained as reported previously[12]. SV40 DNA, labeled with ^{3}H-thymidine, was prepared according to the method of ODA and DULBECCO[13], modified so that extraction with phenol was omitted. A tRNA preparation from *Escherichia coli* K12W6 was a kind gift of Dr. SEKIYA of the Institute. DNA from *Pseudomonas fluorescens* KY4032, prepared according to the method of TOMITA and SUZUKI[14], was kindly supplied by Kyowa-Hakko Co., Ltd., Tokyo.

Strand scission of DNA by bleomycin: Reaction mixtures contained in 200 μl; 50 mM Tris-HCl, pH 7.5 (adjusted at 20°C), SV40 DNA labeled with ^{3}H-thymidine (4×10^{-8} A_{260nm} units, 2,300 cpm), an indicated concentration of bleomycin B_2 or its substitute and, where indicated, $CuSO_4$ or 2-mercaptoethanol. The reaction was initiated by addition of bleomycin (50 μl) and allowed to proceed at 0°C for 30 minutes except where indicated. The reaction was terminated by addition of 10 μl of 5 N NaOH.

Analysis by alkaline sucrose gradient centrifugation: After termination of reaction, the total reaction mixture was gently layered over 4.6 ml of a linear sucrose gradient, 5% to 20% in 3 N NaOH-0.5 M NaCl-10 mM EDTA and centrifuged at 38,000 rpm with a Hitachi RPS-40 rotor (av. 12,000$\times g$) for 4 hours at 4°C in a Hitachi 55P or 65P ultracentrifuge. The centrifuged gradients were pumped out from the bottom to the top and divided into 0.37 ml fractions. Each fraction was mixed with 5 ml of cold 5% TCA solution and left standing for 20 minutes at 0°C. The acid-insoluble material of a fraction was collected on a Millipore filter disc, washed with 3 changes of 5 ml of cold 5% TCA, dried, placed with the filter disc in a counting vial containing 6 ml of scintillation solution (5.0 g of PPO, 0.3 g of dimethyl POPOP and toluene, in 1 liter) and the radioactivity was measured in an Aloka liquid scintillation spectrophotometer (LSC-653).

Fig. 1. Bleomycin action as a function of time: Sedimentation profiles.
Experimental conditions were as described under Methods with the use of 4 μg/ml of bleomycin B_2. The reaction period was varied as follows: 2 minutes (b), 5 minutes (c), 15 minutes (d), 30 minutes (e) and 60 minutes (f). The control (a) received no bleomycin and was kept for 60 minutes. The reaction temperature was 0°C. In a separate experiment (g), a reaction mixture received 4 μg/ml of bleomycin B_2 immediately after pH had been raised above 13 by adding 10 μl of 5 N NaOH and the mixture was kept for about 120 minutes until analysis.



Results

Bleomycin Action as a Function of Reaction Time

The rate of scission of SV40 DNA caused by bleomycin B_2 at a concentration of 4 μg/ml was approximately proportional to reaction time up to 30 minutes (Figs. 1 and 2). The experiments were conducted at 0°C in the absence of any reducing or oxidizing agent. Since we had already found that bleomycin is active at 0°C, conditions were first sought where bleomycin activity could be quickly and completely arrested. As shown in Fig. 1-(g), when the pH of the incubation medium was raised to above 13 prior to addition of bleomycin B_2, the antibiotic did not have an effect at all. Therefore, alkaline pH was employed to terminate the reaction throughout these studies.

Effect of Hydrogen Ion Concentration on Bleomycin Action

As shown in Fig. 3, bleomycin activity was dependent on pH; the optimum pH was 9.1 while no activity was observed below 6 or above 13. The pH-activity curve had 3 distinct maxima.

Fig. 2. Time-course of bleomycin action.
In each of Fig. 1-(a) to Fig. 1-(f), radioactivity in the 53S peak and the 16~18S peak, less background counts, was summed up separately and an index expressing bleomycin activity in each run (R) was calculated: R is the ratio of radioactivity in the 16~18S peak to radioactivity in the 53S peak + radioactivity in the 16~18S peak. In the ordinate in this figure, 0 and 100 represent R of the control run (from Fig. 1-(a)) and the R in the case of total loss of the 53S peak (namely $R=1.0$), respectively.

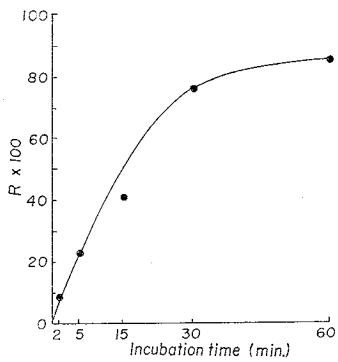
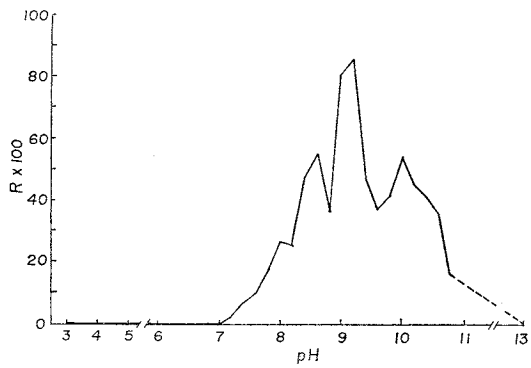


Fig. 3. Effect of hydrogen ion concentrations on bleomycin action.
The reaction mixtures of pH 3 to 8 were based on McLLVAIN's citric acid-phosphate buffered solution (0.1 M citric acid-0.2 M disodium phosphate), while those of pH 8.2~10.8 were based on ATKINS and PANTIN's sodium carbonate-borate buffered solution (0.1 M sodium carbonate-0.1 M borate·potassium chloride). Each reaction mixture (200 μl) consisted of 50 μl of a buffered solution at the desired pH (adjusted at 0°C with a Hitachi-Horiba pH meter, model F-5), 50 μl of bleomycin solution (in the same buffered solution), 20 μl of ^{3}H-SV40 DNA solution (in 1/10 SSC solution-1 mM EDTA, pH 7.0), and 80 μl of water. Otherwise, experiments were conducted following standard conditions.



Effect of Temperature

Bleomycin activity is shown as a function of temperature in Fig. 4. The pH of each reaction mixture was adjusted to 9.1 at the respective temperature. It should be noticed that the reaction proceeded more rapidly at 0°C than 37°C. In the range tested, the optimum temperature was 20°C.

Dependence of Bleomycin Action on its Concentrations

The effect of bleomycin B_2 increased with increasing concentration until a plateau was reached

Fig. 4. Effect of temperature on bleomycin action.
The reaction temperature was varied while the concentration of bleomycin B_2 and the reaction period were fixed at 0.5 μg/ml and 30 minutes, respectively. The pH of reaction mixtures were adjusted to 9.1 (based on ATKINS & PANTIN's buffered solution, see legend to Fig. 3) at each incubation temperature. Other conditions were as described under Methods. A control run (no bleomycin) was conducted at 37°C and its *R* value (see the legend to Fig. 2) was used for 0 on the ordinate. The sedimentation profile of the control run was essentially identical to Fig. 1-(a).

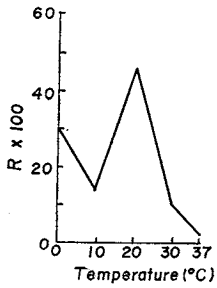
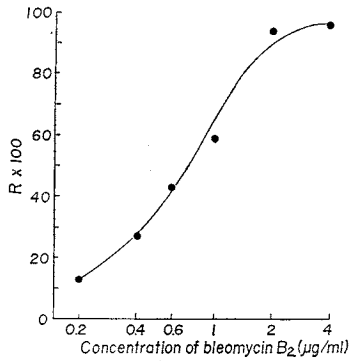


Fig. 5. Dependence of bleomycin action on its concentration.
The effect of bleomycin B_2 at concentrations of 0 (control), 0.2, 0.4, 0.6, 1.0, 2.0, 4.0* and 40* μg/ml were determined under the standard assay condition where the temperature and the reaction time were fixed at 0°C and 30 minutes, respectively. For details, see Methods. Indication of the results are as Fig. 2.
*Conducted as a separate experiment.



at 2 μg/ml, where most DNA molecules were found to have undergone strand scission (Fig. 5). The DNA which had been treated with 2 μg/ml of bleomycin B_2 sedimented as a sharp peak in the 16~18S area, while at extremely high concentrations of bleomycin (40 μg/ml) products sedimenting somewhat slower than 16~18S DNA were formed (Fig. 6). However, the radioactivity in the acid soluble fraction under the latter conditions was still negligible.

Effect of DNA Concentrations on Apparent Activity of Bleomycin

The relative ratio between the concentrations of DNA and bleomycin was critical for the sensitivity of the assay. Upon addition of unlabeled DNA, either of SV40 itself or of other species, to

Fig. 6. Effect of high concentrations of bleomycin B_2 on SV40 DNA.
To obtain a better separation of slower sedimenting products, the centrifugation period was prolonged to 6 hours and the centrifuged solution was divided into 0.28 ml fractions. Otherwise conditions were the same as given in Methods.

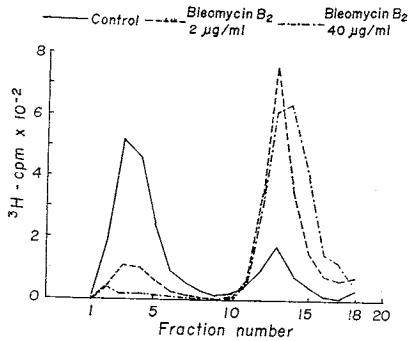
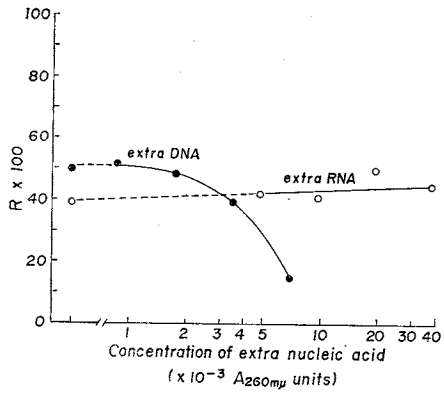


Fig. 7. Effect of DNA or RNA concentration on bleomycin action.
DNA from *Pseudomonas fluorescens* KW4032 or tRNA from *E. coli* K12W6 was added to the reaction mixture at the desired concentration and the reaction was initiated by adding bleomycin B_2 (1 μg/ml) and allowed to proceed at 0° for 30 minutes. Otherwise conditions were the same as given under Methods.



a reaction mixture which contained a fixed amount of ^{3}H-SV40 DNA, the apperent effect of bleomycin B_2 became less. As shown in Fig. 7, 7×10^{-3} A_{260nm} units/200 μl of DNA from *Pseudomonas fluorescens* KY4032 almost protected SV40 DNA from bleomycin action. On the contrary, tRNA from *E. coli* K12W6 exerted no effect on the sensitivity of the assay system even at 3.8×10^{-2} A_{260nm} units/200 μl under the same condition.

Other Characteristics

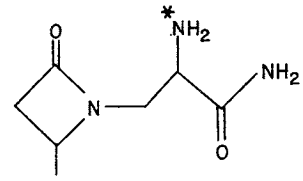
Bleomycin action, observed under the standard assay condition, was stimulated 20-fold by 1 mM 2-mercaptoethanol, since a run with 0.1 μg/ml of bleomycin B_2 in combination with 1 mM 2-mercaptoethanol gave a sedimentation profile resembling the one obtained with 2 μg/ml of bleomycin B_2 alone. Mercaptoethanol at 1 mM did not degrade the DNA when incubated with it for as long as 2 hours. Another experiment revealed that 1 mM $CuSO_4$ almost abolished the effect of 1 μg/ml of bleomycin B_2. This stimulation and inhibition by 2-mercaptoethanol[2,3,4,6] and $CuSO_4$[7], respectively, were as already reported. However, inconsistent with previous reports[4,7], 10 mM EDTA was without effect on bleomycin action in our assay system. It is known that bleomycin is inactivated in various organs. An enzyme responsible for inactivation of bleomycin was partially purified from mouse liver. A product of bleomycin B_2 inactivated by the enzymatic reaction was isolated[12] and subjected to our assay system. Under conditions where 0.7 μg/ml of bleomycin B_2 converted about 50% of 53S DNA to 16~18S DNA, 11 μg/ml of the enzymatically inactivated product was required for the same effect.

Discussion

The preparation of SV40 DNA used in the present study originally included as a minor component the double-stranded, nicked-open-circular DNA (form II), which sediments with S values of 16 and 18 through an alkaline sucrose gradient (Fig. 1-(a)). Bleomycin action also resulted in products sedimenting in the 16~18S region if reaction conditions were restricted to short periods, in the absence of 2-mercatoethanol, and at low concentrations of bleomycin B_2 (Fig. 1-(e)). This indicates that we were observing a reaction which produced a single scission in the total molecule of SV40 DNA. For additional strand scission, a much higher concentration of bleomycin B_2, 40 μg/ml, was required. Under these conditions, the DNA fragments sedimented after the 16~18S region (Fig. 6). SUZUKI *et al.*[7] estimated complex formation between ^{3}H-bleomycin B_2 and DNA and determined the binding ratio to be one molecule of bleomycin B_2 per 350 deoxyribonucleotide residues. In view of the size of SV40 DNA (about 5,000 base pairs, or 10,000 nucleotide residues) and the large excess of bleomycin B_2 to DNA in our reaction mixture, for example 6.6×10^{-7} M (1.0 μg/ml) for the former and 3.3×10^{-10} M for the latter, it seems likely that a considerable number of bleomycin B_2 molecules bind to one molecule of SV40 DNA. Since only a single scission was produced in a SV40 DNA molecule under restricted conditions, the data suggest that multiple bindings of bleomycin B_2 per SV40 molecule lead to conformational distortion which becomes strong enough to produce a strand scission. A nicked-open-circular DNA thus formed, could be rather resistant to another strand scission. The linear time course of bleomycin action (Fig. 2) strongly suggests that bleomycin itself is the active principle capable of producing strand scission in DNA and rules out the mechanism, proposed by NAGAI *et al.*[15], that bleomycin acts only after being activated by some sulfhydryl reagent. In contrast with previous reports[4,7], we find that bleomycin action was not inhibited by EDTA. Those previous incubation mixtures contained 2-mercaptoethanol, and it seemed possible that the sulfhydryl reagent was responsible for this inconsistency. We therefore conducted an experiment in which 2-mercaptoethanol was included in the reactson mixture, but still found no inhibition by EDTA. The simplest explanation for the inhibitory effect of EDTA on bleomycin action, which previous authors observed, could be that EDTA removes a trace amount of Mg^{++}

which is essential for some contaminating nuclease. Such enzymatic cleavage of DNA may become significant if a mixture is incubated at 37°C for a long period.

Bleomycin shows 3 distinct pKá, *i.e.* 2.9, 4.7 and 7.3[16]. The pKá of 7.3 is due to the amino group* of the moiety below. Since bleomycin activity increased markedly in the pH range from 7 to 8, and was negligible below pH 7 (Fig. 3), the unprotonated form of the amino group would appear to be an essential structural component for the bleomycin activity. This view is supported by the fact that when chelated with Cu^{++}, bleomycin lose its ability to cause strand scission of DNA in parallel with the disappearance of the pKá of 7.3. Cu^{++}-chelated bleomycin B_2 (1 atom per molecule) was totally inactive in our assay system. An argument against this mechanism, however, is the fact that for *in vivo* antibacterial and antitumor activities, Cu^{++}-chelated bleomycins are as active as Cu^{++}-free bleomycins[17]. However, bleomycins chelating Cu^{++} could release Cu^{++} ion in cells and act as the free antibiotics. An enzymatically inactivated derivative of bleomycin B_2, which is thought to retain a close resemblance in structure to the parent antibiotic but has slight *in vivo* activity[12], was found much less active in producing a scission in DNA. This seems to support the idea that fragmentation of DNA is of primary importance in the *in vivo* activity of bleomycins.



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