# A ROLE FOR FERROUS ION AND OXYGEN IN THE DEGRADATION OF DNA BY BLEOMYCIN

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Received October 7,1976

SUMMARY: An interaction between bleomycin and low concentrations of Fe(II) in the degradation of DNA is reported. Complete conversion of simian virus 40 DNA to acid-soluble products occurs at approximately equimolar levels of Fe(II), bleomycin, and DNA; Fe(III) does not substitute for Fe(II) in this reaction. Anaerobiosis inhibits the observed DNA degradation by bleomycin and Fe(II). Optical spectral studies reveal that an oxygen-labile complex is formed between bleomycin and Fe(II).

INTRODUCTION: The bleomycins are a family of glycopeptide antibiotics with antineoplastic activity produced by cultures of Streptomyces verticillus. A major activity of the drug is the degradation of cellular DNA (1). Breaks in DNA can be demonstrated in vitro by sucrose gradient centrifugation. DNA breakage is enhanced by relatively high concentrations of organic reducing agents such as ascorbate, dithiothreitol, and 2-mercaptoethanol, but a chemical interaction between bleomycin and these compounds has not been described. The products of the reaction of bleomycin, organic reducing agents and DNA include oligonucleotides and free bases (2,3,4).

It has been reported that the degradation of DNA by bleomycin in the presence of organic reducing agents is enhanced by ATP (5), Fe(II) and Fe(III) (6) and inhibited by the removal of oxygen (7). The effect of Fe(II) and Fe(III) in the absence of organic reducing agents has not been considered. Other studies have suggested an inhibition of bleomycin action in the presence of 2-mercaptoethanol by Cu(II), Zn(II), Co(II) (8,9) and Mg(II) (5). The effect of EDTA is controversial: the chelator has both been reported to inhibit (5,8,9,10) and to have no effect (3,4) on the reaction of bleomycin with DNA.

A persistent problem in understanding the mechanism of bleomycin action has been the role of 2-mercaptoethanol and other organic reducing agents in greatly promoting the drug's effect on DNA. Since bleomycin was first isolated as a copper chelate (11), the possibility occurred to us that adventitious trace metals in the reaction medium would be reduced by organic reducing agents. Metal ion oxidation in an aerobic atmosphere in the presence of bleomycin and DNA could then be instrumental in DNA degradation. Data in this paper are preliminary evidence in support of this thesis.

MATERIALS AND METHODS: Bleomycin $A_2$ (Lot 71L489), a generous gift of Bristol Laboratories, was disolved in deionized water (1-5 mg/ml) and diluted as needed. 2-Mercaptoethanol and disodium EDTA were from Sigma, [$^3$H]thymidine (6.7 Ci/mmole) and scintillant NEN-950 were from New England Nuclear. Oxygen and research grade argon from Linde were purchased from T.W. Smith. Deionized water was used in all experiments. All other chemicals were of reagent grade.

[$^3$H]Simian virus 40[1] DNA was prepared as described by Sambrook and Shatkin (12). The viral DNA was dialyzed against 1 mM Tris pH 8.6 or 3 mM phosphate, pH 7.0 and its concentration estimated by assuming a molar extinction coefficient of $6.6 \times 10^3$ at 260 nm. [$^3$H]HeLa DNA was isolated from nuclei of [$^3$H] thymidine labeled cells. Nuclei were prepared by treatment with detergent NP-40 (Shell) and DNA was purified as described by Marmur (13).

The activity of bleomycin was assayed by determining the proportion of labeled DNA soluble in 0.5 M perchloric acid, as described previously (5). After additions at 4°, assays were conducted for 15 min in stoppered test tubes at 37°. Reactions were initiated by addition of 2-mercaptoethanol or of iron salts in aqueous solution. When both 2-mercaptoethanol and iron were present, reactions were initiated by addition of bleomycin. Fe(II) was added as $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$; Fe(III) was added as $Fe(NH_4)(SO_4)_2 \cdot 12H_2O$. Reactions were terminated by addition of EDTA to a final concentration of 10 mM.

Anaerobiosis was effected by using septum-stoppered test tubes equipped with a sidearm to permit anaerobic mixing of reaction components. Argon equilibrated with an aqueous suspension of phenazine methosulfate and zinc metal was passed through reaction vessels for 20 min at 25° with continuous gentle agitation prior to the start of anaerobic reactions.

Where indicated, reactions terminated by EDTA were analyzed by 5-20% netural sucrose gradients (14). Gradients were centrifuged for 5.75 hrs at 39000 rpm in a Beckman SW41 rotor at 22°, fractionated from the bottom using a peristaltic pump and analyzed for trichloroacetic acid-precipitable material.

Spectroscopic measurements were performed on a Cary 14 R recording spectrophotometer. A thunberg cuvette, equipped with a septum-stoppered sidearm and an optical cell of 10 mm path length was used. Prior to mixing cell and sidearm solutions, an anaerobic atmosphere was achieved using ultra pure argon over phenazine methosulfate and zinc as described above.

[1] SV40

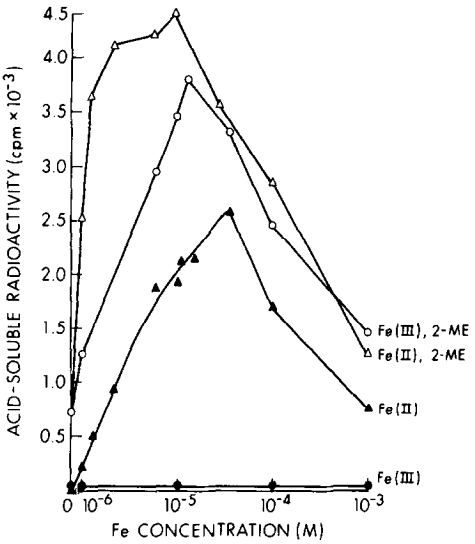


Fig. 1. Effect of iron salts on bleomycin action in the presence or absence of 2-mercaptoethanol (2ME). Reaction mixtures contained in 50 µl: 12.6 µg/ml of [ $^{3}$H]sv40 DNA (4000 cpm), 60 µg/ml of bleomycin, and 50 mM Tris, pH 8.6. These were supplemented by various concentrations of Fe(II) with (▲) or without (▲) 10 mM 2-mercaptoethanol. Other reactions were supplemented with Fe(III) with (○) or without (●) 10 mM 2-mercaptoethanol.

RESULTS: In the presence of 2-mercaptoethanol, bleomycin causes an appreciable portion of the radioactivity in labeled DNA to become acid-soluble. This effect has been described previously (2,6). If the role of the reducing agent is to maintain trace metals in a reduced state, then substantial activity should be observed in the absence of 2-mercaptoethanol if approximately stoichiometric amounts of reduced metal are provided. In the case of iron, Fig. 1 demonstrates that Fe(II) causes marked stimulation of bleomycin action in the absence of organic reducing agent. When 2-mercaptoethanol is present, Fe(II) also imparts considerable augmentation of activity. In sharp contrast, Fe(III) in the absence of 2-mercaptoethanol shows no stimulation of activity, but acts almost as well as Fe(II) when the thiol is present. Reduction of activity at Fe(II) or Fe(III) concentrations greater than $5 \times 10^{-5}$M is variable and is accompanied by the precipitation of iron salts in Tris buffer, pH 8.6. In the absence of bleomycin, Fe(II)

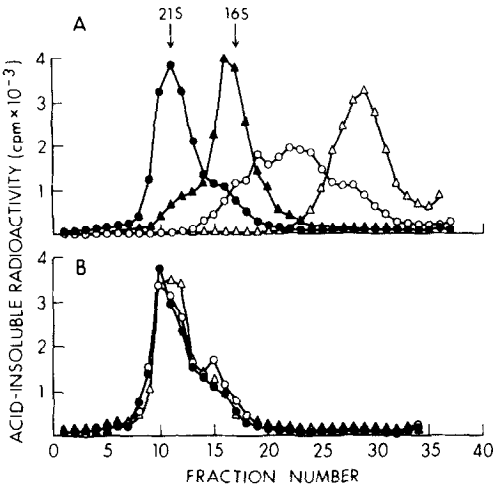


Fig. 2. Effect of Fe(II) and Fe(III) on SV40 DNA in the absence of bleomycin. Neutral sucrose gradient centrifugation analysis. A: Reaction mixtures contained in 100 µl: 50.8 µg/ml of [$^{3}$H]SV40 DNA, 50 mM phosphate pH 7.0 and zero (●), $10^{-5}$M (▲), $10^{-4}$M (○), and $10^{-3}$M (△) Fe(II). B: As in A except zero (●), $10^{-5}$M (▲), and $10^{-4}$M (○) Fe(III). Sedimentation was from right to left.

or Fe(III) causes at most 2% of the DNA to become acid-soluble. EDTA inhibits the degradation of DNA by bleomycin in the presence of 2-mercaptoethanol or added iron. In experiments analogous to those of Fig. 1, Co(II), Mn(II), Cu(II) and Mg(II) do not stimulate the action of bleomycin in the presence or absence of 2-mercaptoethanol.

Since degradation of DNA to acid-soluble products represents the end point of many DNA-breaking events, the effect of Fe(II) on the sedimentation of SV40 DNA was determined. Fig. 2A demonstrates that Fe(II) in the absence of bleomycin is capable of breaking SV40 DNA. At 1 x $10^{-5}$M Fe(II), at least one break per molecule occurs in the SV40 DNA (approximate concentration: 1.5 x $10^{-8}$M in supercoiled molecules) as Form I SV40 DNA (21 S) has been converted largely into a species sedimenting at the position expected (16 S) of the nicked, Form II DNA. Further increases in Fe(II) concentrations are accompanied by a concentration dependent degradation so that at $10^{-3}$M Fe(II), the DNA has been converted to a form sedimenting at about 7 S. Fig. 2B

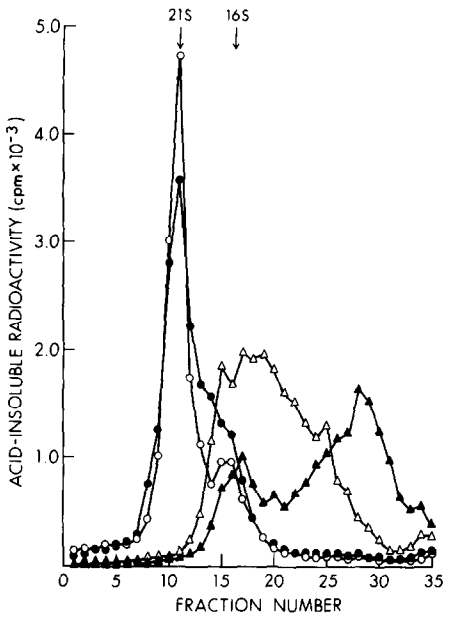


Fig. 3. Breakage of SV40 DNA by bleomycin and Fe(II). Neutral sucrose gradient centrifugation analysis. Reaction mixtures contained in 100 µl: 50.8 µg/ml of [$^3$H]SV40 DNA, 50 mM phosphate, pH 7.0 and the specified concentration of bleomycin and Fe(II): zero bleomycin, zero Fe(II) (●); 40 µg/ml bleomycin, zero Fe(II) (○); 0.4 µg/ml bleomycin, 8 x $10^{-5}$M Fe(II) (▲); and 40 µg/ml bleomycin, 8 x $10^{-5}$M Fe(II) (▲). Sedimentation was from right to left.

clearly demonstrates that Fe(III), at similar concentrations, does not cause even a single break in the vast majority of supercoiled molecules present.

Results summarizing our studies on the effects of bleomycin alone and of bleomycin and Fe(II) on the breakage of SV40 DNA are shown in Fig. 3. Under appropriate conditions, bleomycin and Fe(II) act together to decrease the sedimentation constant of DNA to a greater extent than occurs when either species acts singly. The molar ratio of drug to DNA nucleotide in this experiment is 0.2 or less; at these ratios, acid-soluble radioactivity is not released from labeled DNA.

HeLa DNA was used to determine the effect of oxygen on this reaction. Anaerobiosis inhibits the degradation of DNA by bleomycin and Fe(II)(Fig.4). The effect is most pronounced at low concentrations of bleomycin. At 10 µg/ml

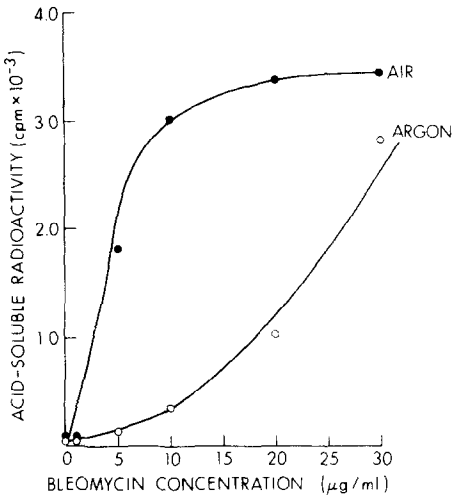


Fig. 4. Effect of anaerobiosis on bleomycin and Fe(II) degradation of HeLa DNA. Reactions contained in a volume of 1.0 ml (after mixing sidearm and main tube contents): 50 mM phosphate buffer, pH 7.0, 0.57 μg/ml of [$^3$H]HeLa DNA (3900 cpm), 1 x $10^{-4}$M Fe(II) and the indicated concentrations of bleomycin. During the removal of air, a 1 x $10^{-3}$M Fe(II) solution in $H_2O$ was in the sidearm and bleomycin, buffer, and DNA were present in the main tube. Reaction was initiated by mixing the solutions in the sidearm and main tube after achieving the desired atmosphere. In the absence of mixing there was no appreciable degradation of DNA after the degassing procedure. Reactions were conducted in the presence of air (●) or deoxygenated argon (○).

bleomycin under anaerobic conditions, the inhibition is almost complete; in contrast, 85% of the DNA was degraded at this concentration in the aerobic reaction. It should be noted that a concentration of 10 μg/ml of bleomycin in this reaction represents a molar ratio of drug to DNA nucleotide of 3-4. At higher concentrations of bleomycin, significant activity by Fe(II) and bleomycin appears even at this level of anaerobiosis.

Optical spectroscopy demonstrates that a complex is formed between bleomycin and Fe(II). The complex, studied under anaerobic conditions, exhibits a broad maximum near 480 nm and another, smaller absorption near 370 nm (Fig. 5B). Bubbling oxygen through both reference and sample solutions changes the spectrum ascribed to the Fe(II)-bleomycin complex to one in which the absorptions near 480 and 370 nm have disappeared,

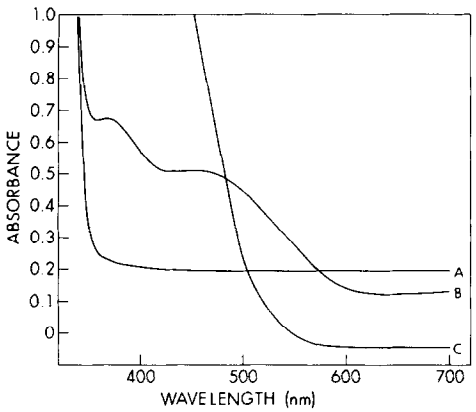


Fig. 5. Spectral studies of Fe(II)-bleomycin. Thunberg cuvettes contained the following solutions. Reference: 3 ml of 50 mM phosphate, pH 7.0; Sample: 3 ml of approximately 2 x $10^{-3}$M of bleomycin (3.1 mg/ml) in 50 mM phosphate. The sidearm of both cuvettes contained 60 µl of 1 x $10^{-1}$M Fe(II). The real baseline (A=0) was set at 0.2 on the chart. Curve A: spectrum in the presence or absence of air prior to mixing of cuvette and side arm contents; Curve B: spectrum in the absence of air after mixing; Curve C: spectrum of B after bubbling $O_2$ through reference and sample solutions for 3 min at 25°. The decrease in baseline absorbance on adding Fe(II) to reference and sample compartments can be attributed to the greater turbidity due to iron phosphate precipitation that develops in the reference compartment in the absence of chelator.

leaving an intense absorption in the near-ultraviolet. The addition of dithionite then restores the spectrum to one with an absorption at 480 nm. These spectral studies demonstrate that under conditions where Fe(II) and bleomycin are active in degrading DNA, a complex is formed between the two species, the stability of which is sensitive to oxygen.

DISCUSSION: It has long been known that Fe(II) is injurious to DNA (15,16). What is novel in the present experiments is the specific interaction of bleomycin and Fe(II) in causing extensive degradation of DNA in the absence of organic reducing agents. This reaction, which is stimulated by oxygen, is correlated with an oxygen-mediated spectroscopic change in a bleomycin-Fe(II) complex. The concentrations at which Fe(II) exhibits its effect in the bleomycin mediated degradation of DNA are 2-3 orders of magnitude lower

than those at which, for example, 2-mercaptoethanol stimulates bleomycin activity maximally (3).

These considerations, as well as the observation that EDTA inhibits the breakage of DNA by bleomycin and organic reducing agents (5), prompt us to consider a role for trace iron in reactions of bleomycin to which exogenous iron has not been added. Preliminary experiments, including atomic absorption analysis of the reagents used in one such reaction, that of bleomycin, 2-mercaptoethanol and ATP (5) are consistent with the idea that ATP-mediated stimulation of bleomycin activity may be due to the presence of iron impurities. Substantiation of this idea is derived from inhibition studies which show that deferoxamine mesylate, a very potent iron chelator, inhibits the ATP-mediated reaction at concentrations 2-3 orders of magnitude lower than those at which EDTA inhibits this reaction. Further experiments are necessary to clarify the relationship of the Fe(II)-bleomycin reaction to the other reactions of bleomycin that have been described (2-10).

ACKNOWLEDGEMENTS: The authors thank Drs. M. Horwitz and A.P. Grollman for their helpful discussions.

This work was supported in part by United States Public Health Service Grants CA 15714 ( S.B.H.) and HL-13399 (J.P.). E.A.S. is a Medical Scientist Trainee of the United States Public Health Service, Grant 5T5 GM 1674. S.B.H. is a recipient of an Irma T. Hirschl Career Scientist Award.

REFERENCES:

1. Umezawa, H. (1975) in Antibiotics III, ed., Corcoran, J.W. and Hahn, F.E. (Springer-Verlag, Berlin), pp. 21-33.
2. Haidle, C.W. (1972) Mol. Pharmacol. 8, 531-537.
3. Müller, W.E.G., Yamazaki, Z., Breter, H. and Zahn, R.K. (1972) Eur. J. Biochem. 31, 518-525.
4. Umezawa, H., Akasura, H., Oda, K. and Hori, S. (1973) J. Antibiotics 26, 521-527.
5. Takeshita, M., Grollman, A.P. and Horwitz, S.B. (1976) Virology 69, 453-463.
6. Ishida, R. and Takahashi, T. (1975) Biochem. Biophys. Res. Commun. 66, 1432-1438.
7. Onishi, T., Iwata, H. and Takagi, Y. (1975) J. Biochem. 77, 745-752.

8. Nagai, K., Yamaki, H., Suzuki, H., Tanaka, N. and Umezawa, H. (1969) Biochem. Biophys. Acta 179, 165-171.
9. Suzuki, H., Nagai, K., Akutsu, E., Yamaki, H., Tanaka, N. and Umezawa, H. (1970) J. Antibiotics 23, 473-480.
10. Shirakawa, I., Azegami, M., Ishii, S. and Umezawa, H. (1971) J. Antibiotics 24, 761-766.
11. Umezawa, H., Maeda, K., Takeuchi, T. and Okami, Y. (1966) J. Antibiotics Ser. A 19, 200-209.
12. Sambrook, J. and Shatkin, A.J. (1969) J. Virol. 4, 719-726.
13. Marmur, J. (1961) J. Mol. Biol. 3, 208-218.
14. Beerman, T. and Goldberg, I.H. (1974) Biochem. Biophys. Res. Commun. 59, 1254-1261.
15. Zamenhof, S., Griboff, G. and Marullo, N. (1954) Biochim. Biophys. Acta 13, 459-470.
16. Vinograd, J., Lebowitz, J., Radloff, R., Watson, R. and Laipis, P. (1965) Proc. Nat. Acad. Sci. USA 53, 1104-1111.