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Effect of Chelating Agents and Metal Ions on the Degradation of DNA by Bleomycin†

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ABSTRACT: The degradation of DNA by bleomycin was studied in the absence and in the presence of added reducing agents, including 2-mercaptoethanol, dithiothreitol, reduced nicotinamide adenine dinucleotide phosphate, H_2O_2, and ascorbate, and in the presence of a superoxide anion generating system consisting of xanthine oxidase and hypoxanthine. In all cases, breakage of DNA was inhibited by low concentrations of chelators; where examined in detail, deferoxamine mesylate was considerably more potent than (ethylenedinitrilo)tetraacetic acid. Iron was found to be present in significant quantities in all reaction mixtures. Thus, the pattern of inhibition observed is attributed to the involvement of contaminating iron in the degradation of DNA by bleomycin. Cu(II), Zn(II), and Co(II) inhibit degradation of DNA by bleomycin and Fe(II) in the absence of added reducing agents. A model is proposed in which the degradation of DNA in these systems is dependent on the oxidation of an Fe(II)–bleomycin–DNA complex.

Bleomycin is a glycopeptide antibiotic used for the treatment of selected human neoplastic diseases (Blum et al., 1973). It was originally obtained by Umezawa et al. (1966a,b) as a copper chelate from culture filtrates of *Streptomyces verticillus*. As isolated, bleomycin consists of a group of glycopeptides differing only in a terminal cationic or amine functional group (Umezawa, 1974). The structure of bleomycin A_2, the principal component of the clinically used preparation, is shown in Figure 1. In cultured cells, a major effect of bleomycin is the introduction of strand breaks into DNA (Suzuki et al., 1969; Takeshita et al., 1974). Similar breakage may be observed in isolated DNA incubated with the drug, but efficient breakage of DNA by bleomycin has been reported to require the presence of reducing agents, such as 2-mercaptoethanol, dithiothreitol, ascorbate, and hydrogen peroxide (Suzuki et al., 1969; Onishi et al., 1975). Although reducing agents alone are injurious to DNA (Bode, 1967; Rhaese and Freese, 1967), the combination of bleomycin with a reducing agent is far more effective in degrading DNA than is either bleomycin or reducing agent alone (Shirakawa et al., 1971; Umezawa et al., 1973). Oligonucleotides, free bases, and aldehyde moieties are found in limit digests of DNA in the presence of bleomycin and with either 2-mercaptoethanol or dithiothreitol (Haidle, 1971; Haidle et al., 1972; Müller et al., 1972; Kuo and Haidle, 1974). Limited damage to DNA may be observed with high concentrations of bleomycin alone (Umezawa, 1973; Haidle, 1971).

Since bleomycin A_2 binds to DNA in the absence of added reducing agents (Chien et al., 1977), the role of the latter compounds in promoting the degradation of DNA by bleomycin has been studied intensively in this laboratory. We have established that Fe(II) can substitute for the reducing agents previously reported to promote highly efficient degradation of DNA with bleomycin. Bleomycin and Fe(II) together are far more efficient in cleaving DNA than either species alone (Sausville et al., 1976). This result has recently been verified by Lown and Sim (1977).

We have suggested that the formation of an oxygen-labile complex between Fe(II) and bleomycin is related to DNA cleavage. Fe(III) cannot replace Fe(II) in the degradation of DNA with bleomycin, but in the presence of a reducing agent either Fe(II) or Fe(III) greatly stimulates DNA degradation by bleomycin.

In the presence of organic reducing agents, EDTA,[1] at relatively high concentrations, can inhibit the degradation of DNA by bleomycin in solutions to which no metal ion had been added (Suzuki et al., 1970; Shirakawa et al., 1971; Takeshita et al., 1976; Bearden et al., 1977). A detailed study of the effect of chelating agents on the action of bleomycin under various conditions has not been conducted. Such an investigation as-

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‡ A Medical Scientist Trainee of the United States Public Health Service, Grant 5T 32 GM 7288.
§ A recipient of an Irma T. Hirschl Career Scientist Award.

[1] Abbreviations used: Tris, tris(hydroxymethyl)aminomethane; EDTA, (ethylenedinitrilo)tetraacetic acid; SV40, Simian virus 40; NMR, nuclear magnetic resonance.

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sumes importance in view of the capacity of Fe(II) to act in concert with bleomycin to degrade DNA and in view of the ability of many reducing agents commonly used with bleomycin to generate Fe(II) from Fe(III). The results of our study indicate that in the presence of reducing agents it is the interaction between bleomycin and contaminating iron which causes cleavage of DNA by bleomycin. According to this view, the cleavage of DNA observed in the presence of bleomycin and reducing agents depends upon the reduction of adventitious Fe(III) to Fe(II), which then acts together with bleomycin to cause the oxidative degradation of DNA.

Materials and Methods

Chemicals. Bleomycin was obtained as a gift from Bristol Laboratories. Our preparations of bleomycin consist of 70% bleomycin A_2 and approximately 30% bleomycin B_2. Bleomycin B_2 contains agmatine in place of the 3-amino-propyldimethylsulfonium residue of bleomycin A_2. Where these two congeners have been examined, biologic activities are identical. Chen et al. (1977) found by NMR spectroscopy that these congeners have very similar conformations. Calculations of molar concentrations of drug were based on a molecular weight of 1550. Bleomycin was dissolved in deionized water immediately prior to use. Deferoxamine mesylate was a generous gift of Ciba-Geigy. [*methyl*-^{3}H]Thymidine (40–60 Ci/mmol) was purchased from ICN and diluted to 16 Ci/mmol prior to use. ATP, ADP, and calf thymus DNA were from Worthington; dithiothreitol, NADPH, Tris, 2-mercaptoethanol, EDTA, xanthine oxidase (xanthine:oxygen oxidoreductase, EC 1.2.3.2), catalase (H_2O_2:H_2O_2 oxidoreductase, EC 1.11.1.6), and superoxide dismutase ($\cdot O_2^-$:O_2^- oxidoreductase, EC 1.15.1.1) were from Sigma; L-ascorbic acid, H_2O_2, sodium phosphates, and $ZnCl_2$ were from Fisher; $Cu(C_2H_3O_2)_2 \cdot H_2O$ was from Baker; $CoCl_2 \cdot 6H_2O$ was from British Drug Houses; and $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ and $Fe(NH_4)(SO_4)_2 \cdot 12H_2O$ were Mallinkrodt analytical reagents.

DNA. SV40 [^{3}H]DNA was prepared as described by Sambrook and Shatkin (1969). Adenovirus type 2 [^{3}H]DNA was isolated from the virion as described by Horwitz (1971). DNAs were dialyzed against 2 mM Tris-HCl, pH 7.5, except where indicated, and the concentration of DNA was estimated by assuming an extinction coefficient of 6.6×10^3 M^{-1} at 260 nm.

Assay for DNA Breakage. The degradation of radioactively labeled DNA by bleomycin was assayed by following the production of acid-soluble radioactivity (Takeshita et al., 1976). After preparation and incubation as described in table and figure legends, all reactions were terminated by the addition of EDTA to a final concentration of 0.05 M, and a portion of the acid-soluble fraction was counted in 8 mL of Yorktown TT-21 scintillant. Alternatively, reaction mixtures were layered on 5–20% (4.8 mL) alkaline sucrose gradients which were centrifuged at 40 000 rpm for 155 min at 22 °C in a Beckman SW 50.1 rotor. Fractions were collected from the bottom of the sucrose gradients, and radioactivity was determined as described (Poon et al., 1977).

Analysis of Fe in Reaction Mixtures. The concentration of Fe in representative reaction mixtures was determined by atomic absorption with a Varian Techtron Type AA5 spectrophotometer, which was equipped with a CRA-90 carbon rod atomizer and a Varian Fe lamp (no. EV004). The carbon cup held a sample of 5 μL, which was dried at 100 °C for 30 s, ashed at 1200 °C for 30 s, and atomized at 2200 °C for 1.5 s; the atomizing temperature was attained 2.5 s after completion of the ashing period. The intensity of the absorbance peak in the atomizing cycle was recorded. Standard Fe solutions, prepared in deionized glass-distilled water, contained the EDTA complex of iron from $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ in 0.01 M HNO_3 (Baker, Ultrex). Samples were never more concentrated than 0.05 M in phosphate. If chloride was present, HNO_3 was added in fivefold molar excess.

BLEOMYCIN A_2

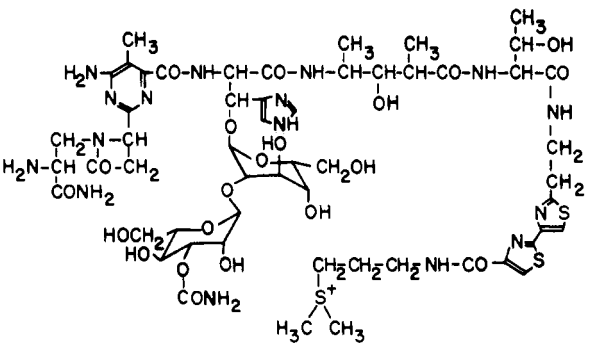

FIGURE 1: Structure of bleomycin A_2.

Results

Degradation of DNA by Bleomycin in the Absence of Reducing Agents. Figure 2A demonstrates that bleomycin causes the breakage of adenovirus-2 DNA in the absence of added reducing agents. Bleomycin at a concentration of 10 μg/mL has little effect on DNA during a 15-min incubation, whereas at a drug concentration of 50 μg/mL breakage of DNA occurs. In Figure 2B, 10^{-5} M EDTA does not inhibit DNA cleavage using 50 μg/mL bleomycin, but 10^{-3} M EDTA markedly diminishes the extent of this reaction. Deferoxamine, a naturally occurring polyhydroxamic acid, is an extremely strong and highly specific chelator for Fe(III) (Sillen and Martell, 1964; Emery, 1971). If contaminating Fe is related to the degradation of DNA by bleomycin in this reaction, then more efficient inhibition of DNA breakage should be observed with deferoxamine than with equimolar concentrations of EDTA. Figure 2C demonstrates that, in contrast to 10^{-5} M EDTA, 10^{-5} M deferoxamine affords substantial protection to DNA in the presence of 50 μg/mL bleomycin.

Degradation of DNA by Bleomycin in the Presence of Reducing Agents. 2-Mercaptoethanol has been used with bleomycin to cause the degradation of DNA to acid-soluble products, including oligonucleotides and free bases (Haidle et al., 1972; Kuo and Haidle, 1974; Ishida and Takahashi, 1975). More recently, Mg(II)–ATP has been shown to stimulate this reaction by two- to tenfold (depending upon reaction conditions) via a mechanism that does not involve the hydrolysis of ATP (Takeshita et al., 1976, 1977). We have examined the effect of chelators on this reaction in detail. In Figure 3A, deferoxamine, at a concentration of 10^{-6} M, inhibits by 50% the degradation of adenovirus-2 DNA by bleomycin in the presence of 2-mercaptoethanol. In contrast, EDTA inhibits the reaction to the same extent, but only at a tenfold higher concentration. Figure 3B demonstrates an analogous effect on the degradation of DNA by bleomycin in the presence of both 2-mercaptoethanol and Mg(II)–ATP. Again, EDTA is not as efficient an inhibitor as deferoxamine and about a tenfold higher concentration of EDTA in relation to deferoxamine is required. In Figure 3C, SV40 DNA and bleomycin are employed in an analogous reaction to Figure 3B, but containing eight- and tenfold higher concentrations of SV40 DNA and bleomycin, respectively, than the adenovirus-2 DNA and bleomycin concentrations used in Figure 3B. Def-

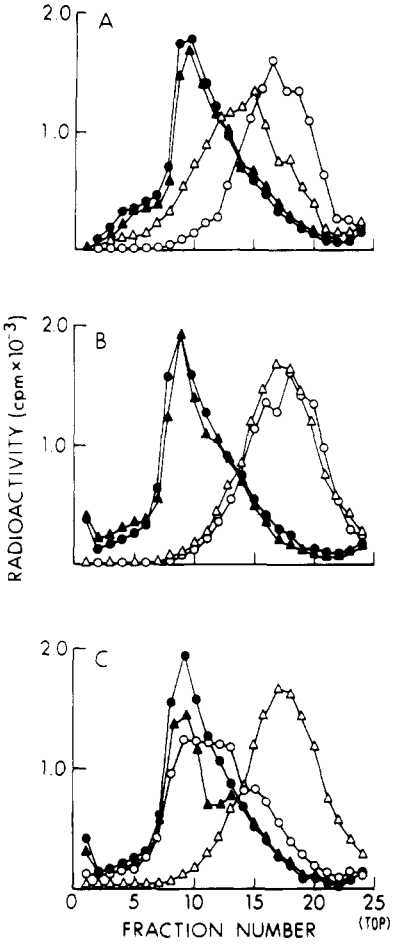

FIGURE 2: Effect of chelators on the degradation of DNA by bleomycin in the absence of reducing agents. (A) Reaction mixtures contained, in a final volume of 50 μL, 21 μM adenovirus [^{3}H]DNA, 0.05 M phosphate buffer, pH 7.0, and bleomycin in the following concentrations: 0 (●), 10 (▲), 30 (△) and 50 μg/mL (○). Bleomycin was the last addition. Incubation was for 15 min at 37 °C. Reaction mixtures were centrifuged in alkaline sucrose gradients. Sedimentation is from right to left. (B) As in A, except reactions contained 20 μM adenovirus [^{3}H]DNA and 0 μg/mL bleomycin (●), 50 μg/mL bleomycin (△), 50 μg/mL bleomycin plus 10^{-5} M EDTA (○), and 50 μg/mL bleomycin plus 10^{-3} M EDTA (▲). (C) Reactions as in B, except that deferoxamine was substituted for EDTA. It should be noted that the concentration of DNA in Figure 2A,B,C is approximately 3×10^{-10} M in full-length adenovirus-2 DNA molecules.

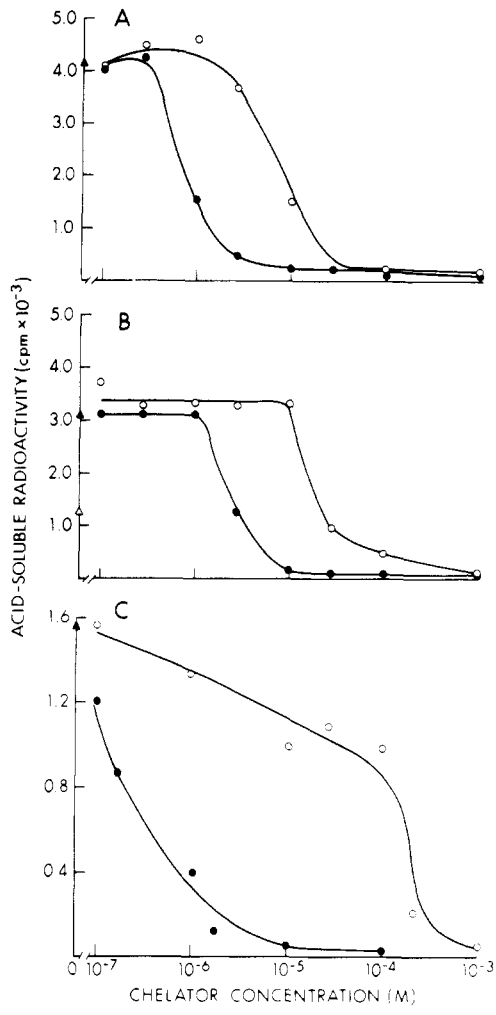

FIGURE 3: Effect of chelators on the degradation of DNA by bleomycin in the presence of reducing agents. (A) Reaction mixtures contained in a final volume of 50 μL: 5 μM adenovirus [^{3}H]DNA (5900 cpm), 0.05 M Tris-HCl, pH 8.6, 40 μg/mL bleomycin, 0.01 M 2-mercaptoethanol, and the indicated concentrations of EDTA (○) or deferoxamine (●). A reaction conducted in the absence of chelator is indicated (▲). In all cases, 2-mercaptoethanol was the last addition. The amount of acid-soluble radioactivity was determined after incubation for 15 min at 37 °C. (B) Reactions as in A, except 1 mM ATP and 1 mM $MgCl_2$ were added while bleomycin was present at a concentration of 10 μg/mL. Reactions which proceeded in the absence of chelator but in the presence (▲) or absence (△) of ATP and $MgCl_2$ are indicated. (C) Reaction mixtures contained in a volume of 50 μL: 38 μM of SV40 [^{3}H]DNA (3400 cpm), 0.05 M Tris-HCl, pH 8.6, 1 mM ATP, 1 mM $MgCl_2$, 100 μg/mL bleomycin, 0.01 M 2-mercaptoethanol, and the indicated concentration of EDTA (○) or deferoxamine (●). Bleomycin was the last addition. DNA was dialyzed against 3 mM phosphate buffer, pH 7.0, prior to use. Incubation was for 15 min at 37 °C. A reaction conducted in the absence of chelator is indicated (▲).

eroxamine causes 50% inhibition of the reaction at a chelator concentration that is two to three orders of magnitude lower than that necessary for a similar effect using EDTA.

In addition to 2-mercaptoethanol, other reducing agents are known to promote the efficient degradation of DNA by bleomycin (Onishi et al., 1975). The effect of chelating agents on the action of bleomycin is examined in Table I. Dithiothreitol, NADPH, ascorbate, and H_2O_2 are all capable of acting with bleomycin to cause the degradation of adenovirus DNA to acid-soluble products. In the absence of the antibiotic, some of these compounds, especially ascorbate, cause limited damage. Except for the case where reductant is omitted, bleomycin converts up to 100% of the DNA to an acid-soluble form. When 5 mM EDTA is present with bleomycin in reaction mixtures, the degradation of DNA is markedly decreased. Deferoxamine at a concentration of 1 mM is at least as effective as 5 mM EDTA in inhibiting the reaction. In addition, under conditions analogous to those of Table I but in the presence of 2-mercaptoethanol, 1 mM each of the known iron chelators, 8-hydroxyquinoline 5-sulfonic acid, α,α′-dipyridyl, and 4,5-dihydroxy-*m*-benzenedisulfonic acid (Tiron), inhibited the degradation of DNA by 80–95%.

Degradation of DNA by Bleomycin in the Presence of a Superoxide-Generating System. Ishida and Takahashi (1975) reported that the degradation of DNA by bleomycin was enhanced by the presence of xanthine oxidase and hypoxanthine. They presented evidence suggesting that $\cdot O_2^-$, superoxide anion, which is generated by this system (McCord and Fridovich, 1968), is responsible for this effect. However, since Fe(II) can be generated from the reaction of Fe(III) with the $\cdot O_2^-$ produced by xanthine oxidase and hypoxanthine (Fong et al., 1976), there is a basis for the involvement of Fe(II) in the action of bleomycin in the presence of xanthine oxidase. The experiments presented in Table II explore this possibility.

TABLE I: Effect of Chelators on the Degradation of DNA by Bleomycin and Reducing Agents.[a]

reductant	acid-soluble radioactivity (cpm)			
	0 bleomycin	100 μg/mL bleomycin	100 μg/mL bleomycin, 5 mM EDTA	100 μg/mL bleomycin, 1 mM deferoxamine
None	93	91		
5 mM DTT	65	10 500	241	200
10 mM NADPH	110	1 450	180	77
10 mM ascorbate	1700	12 000	1500	600
10 mM H_2O_2	280	9 500	1400	590

[a] Reactions were conducted in a final volume of 50 μL and contained 0.05 M Tris-HCl, pH 8.0, 12 μM of adenovirus [^{3}H]DNA (11 600 cpm), and bleomycin, chelators, and reducing agents as indicated. All components were equilibrated at 37 °C for 3 min prior to addition of reducing agent. Incubation was for 15 min at 37 °C.

TABLE II: Degradation of DNA by Bleomycin in the Presence of Xanthine Oxidase and Hypoxanthine.[a]

conditions	acid-soluble radioact. (cpm)
complete	440
minus bleomycin	36
minus hypoxanthine	84
minus bleomycin and hypoxanthine	48
plus 20 μg/mL superoxide dismutase	74
plus 50 μg/mL catalase	1020
plus 50 μg/mL dialyzed catalase	1130
plus 10^{-3} M EDTA	95
plus 10^{-6} M EDTA	400
plus 10^{-6} M deferoxamine	270
plus 5×10^{-5} M Fe(III)	1600
plus 5×10^{-5} M Fe(III), 1×10^{-4} M ADP	2500

[a] The complete reaction contained in a volume of 50 μL: 0.05 M Tris-HCl, pH 8.0, 60 μg/mL bleomycin; 31 μM SV-40 [^{3}H]DNA (5000 cpm); 50 μg/mL xanthine oxidase, and 1×10^{-4} M hypoxanthine. The reaction was prepared at 4 °C (bleomycin added last); incubation was for 30 min at 37 °C.

TABLE III: Inhibition by Metal Ions of the Degradation of DNA by Bleomycin and Fe(II).[a]

conditions	acid-soluble radioact. (cpm)
complete reaction mixture	2540
minus bleomycin	41
minus Fe(II)	43
minus bleomycin and Fe(II)	39
plus 5×10^{-5} M Cu(II)	36
plus 5×10^{-5} M Zn(II)	45
plus 5×10^{-5} M Co(II)	32
plus 5×10^{-5} M Fe(III)	1720
minus Fe(II), plus 10^{-5} M 2-mercaptoethanol	140
minus Fe(II), plus 10^{-5} M ascorbate	92
minus Fe(II), plus 10^{-5} M H_2O_2	84

[a] The complete reaction mixture contained in a final volume of 50 μL: 0.05 M phosphate, pH 7, 5 μM adenovirus-2 [^{3}H]DNA (5800 cpm), 50 μg/mL bleomycin, and 1×10^{-5} M Fe(II). Reactions were initiated by addition of Fe(II) at 37 °C and incubation continued for 15 min.

It can be seen that the degradation of DNA by bleomycin in the presence of xanthine oxidase and hypoxanthine is dependent upon both the antibiotic and the enzyme under conditions where superoxide is generated. Superoxide dismutase inhibits this reaction, while catalase enhances it, as was observed by Ishida and Takahashi (1975). In addition, our experiments demonstrate that EDTA, at 10^{-3} but not at 10^{-6} M, is capable of inhibiting the reaction. In contrast to EDTA, 10^{-6} M deferoxamine causes a greater inhibitory effect. Since these chelators neither inhibit superoxide production, as measured by the aerobic action of xanthine oxidase and hypoxanthine to reduce cytochrome *c*, nor the production of uric acid from hypoxanthine (data not shown), these results suggest that exchangeable Fe is involved in the degradation of DNA by bleomycin in the presence of this superoxide-generating system. As would be expected from this result, added Fe(III) stimulates the degradation of DNA seen in this system in a way that recalls the stimulation of DNA degradation by Fe(III) when added to bleomycin in the presence of 2-mercaptoethanol (Ishida and Takahashi, 1975; Sausville et al., 1976). ADP added with Fe(III) increases this stimulation to an even greater extent, reminiscent of the stimulation by nucleoside phosphates of the degradation of DNA by bleomycin in the presence of 2-mercaptoethanol (Takeshita et al., 1976). It should be pointed out that neither nucleoside phosphates nor Fe(III) added to reactions containing bleomycin and DNA in the absence of reducing agents acts to cause the breakage of DNA (Sausville et al., 1976; Takeshita et al., 1976). Catalase itself similarly does not act to degrade DNA and has in fact been used to inhibit the degradation of DNA caused by streptonigrin (Cone et al., 1976).

Effect of Metal Ions on the Degradation of DNA by Bleomycin and Fe(II). It has been reported that the action of bleomycin on DNA in the presence of 2-mercaptoethanol is inhibited by Cu(II), Zn(II), and Co(II) (Nagai et al., 1969). If, in reactions containing reducing agents, adventitious Fe(II) is the species which directly acts with bleomycin to degrade DNA, then these metal ions should be able to diminish effectively the action of added Fe(II) with bleomycin, in the absence of reducing agents. The experiments presented in Table III demonstrate that Cu(II), Zn(II) and Co(II), present at fivefold molar excess in relation to Fe(II), completely abolish the activity seen in their absence. Exogenously added Fe(III) is not as effective an inhibitor. In contrast to Fe(II), these metal ions do not act with bleomycin to degrade DNA. The experiments in Table III also indicate that on a molar basis Fe(II) is far more effective than 2-mercaptoethanol, ascorbate, or H_2O_2 in acting with bleomycin to degrade DNA. The fact that Fe(III) is not as effective an inhibitor of the reaction as Cu(II), Co(II), and Zn(II) may extend from either the limited solubility of Fe(III) when added to these reaction mixtures, an intrinsically lower affinity of bleomycin for Fe(III), or a difference in the binding site of Fe(III) as compared to other metal ions.

Analysis for Fe. Table IV presents the results of chemical

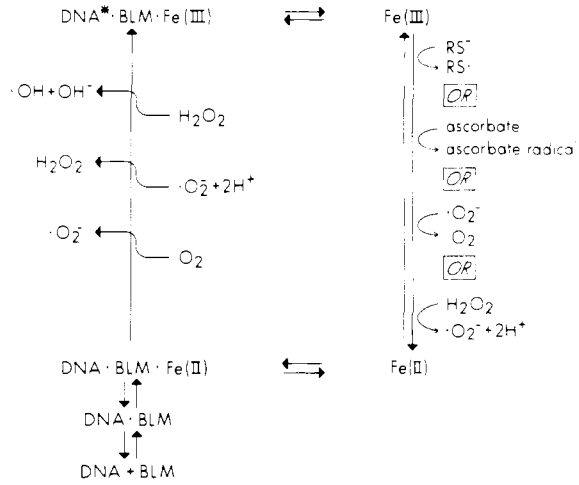

FIGURE 4: Model proposed for the degradation of DNA by bleomycin. The antibiotic (BLM) binds to DNA. Fe(II) combines with BLM either before or after it binds to DNA. Oxidation of DNA-BLM-Fe(II) ultimately results in damaged DNA (DNA*). Fe(III) can be reduced to Fe(II) in the presence of a number of reducing agents and thereby take part in many DNA-breaking events. The reduction of Fe(III) shown here does not presuppose a specific site of binding of the metal ion during its reduction to Fe(II).

analyses for Fe content conducted on solutions in which bleomycin is active in causing the degradation of DNA. Although the solutions analyzed contained, in general, a tenfold greater concentration of reactants than was employed in assays for bleomycin activity, it is nonetheless apparent that Fe is present in significant quantities in all of these samples. The source of the Fe was not determined. It is recognized that all of the Fe present may not be available to participate in the degradation of DNA by bleomycin. This would be expected, for example, in reactions employing the superoxide-generating system, in which Fe is a tightly bound metal cofactor of xanthine oxidase.

It should be cautioned that a linear relationship relating Fe content to activity in degrading DNA in our systems is not observed. This is not surprising in view of the propensity of Fe to associate tightly with the components of these reaction mixtures, especially nucleic acids (Wacker and Vallee, 1959).

Discussion

Bleomycin is a chelating agent which binds to DNA (Nunn, 1976; Chien, 1977). We propose that the ability of bleomycin to bind metal ions is intimately related to the degradation of DNA in vitro by this antibiotic. Specifically, we believe that Fe(II) is an ultimate cofactor of bleomycin in the breakage of DNA as commonly observed in the presence or absence of added reducing agents. The evidence for this is as follows. Fe is present in significant quantities in reaction mixtures where bleomycin is active in degrading DNA (Table IV); Fe(II) but not Fe(III) can act with bleomycin to effect the highly efficient degradation of DNA; other metal ions which are known to form complexes with bleomycin inhibit this reaction, as well as the degradation of DNA observed in the absence of added Fe(II) (Table III; Nagai et al., 1969). In the presence of 2-mercaptoethanol, both Fe(II) and Fe(III) act to stimulate the degradation of DNA that is seen in the absence of added Fe. Fe(II) and bleomycin form an oxygen-labile complex; the degradation of DNA by bleomycin and Fe(II) is oxygen-dependent (Sausville et al., 1976, 1978). The degradation of DNA by bleomycin in the absence of either added Fe or a reducing agent is extremely limited by comparison to that seen in the presence of a reducing agent (Figure 2, Table I). All

TABLE IV: Analysis of Fe Present in Reaction Mixtures.[a]

Sample	Composition	Fe (μM)
A	71 μg/mL DNA, 500 μg/mL bleomycin, 0.05 M phosphate, pH 7.0	22
B	react. mix. (10×) from Figure 3A	55
C	react. mix. (10×) from Figure 3B	120
D	react. mix. (10×) from Figure 3C	240
E	react. mix. (10×) of Table I in the presence of bleomycin and ascorbate	210
F	complete react. mix. (10×) of Table II	1300

[a] Samples of reaction mixtures were used for Fe analysis by atomic absorption spectroscopy. Calf thymus DNA was substituted for radioactive viral DNA, and chelators were omitted. It should be noted that xanthine oxidase, a metalloenzyme containing iron, is probably responsible for the extremely high Fe content of sample F.

reactions of bleomycin leading to the degradation of DNA in the presence or absence of reducing agents are inhibited by chelating agents. Where the specificity of this effect has been examined in detail, deferoxamine, a specific iron chelator, is considerably more effective in inhibiting the reaction than is EDTA, which binds Fe much less selectively (Sillen and Martell, 1964) (Figures 2 and 3, Tables I and II).

We therefore propose the scheme shown in Figure 4 as a plausible mechanism for the degradation of DNA by bleomycin. Bleomycin can bind to DNA in the absence of metal ion or added reducing agent. Bleomycin binds Fe(II) and thus a ternary complex may then be formed. Oxidation of Fe(II) by oxygen or one of its reduced forms can then occur, producing a variety of potentially reactive free-radical species. These radical species are proposed to participate in the degradation of DNA. It should be noted that there is no direct evidence that any of the reduced forms of oxygen shown in Figure 4 participate in the oxidation of Fe(II)-bleomycin. It is clear only that molecular oxygen is required for the highly efficient degradation of DNA to produce acid-soluble products (Sausville et al., 1978). We recognize that the binding of oxygen to Fe(II)-bleomycin or to Fe(II)-bleomycin-DNA could be an important aspect of the role of oxygen in DNA degradation. Fe(III) generated by the oxidation of Fe(II)-bleomycin may undergo reduction by a reducing agent, if present, and thus be available for further action with bleomycin. This step is required in cases in which Fe(II) has not been added and where adventitious Fe is utilized in the reaction.

Several features of this mechanism require comment. Interference in the action of Fe(II)-bleomycin by Co(II), Cu(II), and Zn(II) is proposed to arise from the complexation of these metal ions with bleomycin, leading to an exclusion of Fe(II). The fact that Fe(II), Co(II), Cu(II), and Zn(II) produce similar perturbations of the ultraviolet spectrum of bleomycin (unpublished results) is evidence that these metal ions bind at the same or a similar site as does Fe(II). Despite the ability of various metal ions to form complexes with bleomycin, of those ions thus far examined, only Fe(II) has significant activity in the absence of reducing agents, while only Fe(II) and Fe(III) stimulate the degradation of DNA observed in the presence of reducing agents. It is conceivable though that other metal ions may be found to act with bleomycin to degrade DNA by an analogous redox mechanism.

There is ambiguity in assigning specific roles for O_2 and its reduced forms, $\cdot O_2^-$, H_2O_2, and $\cdot OH$, in the degradation of DNA by bleomycin. All are potentially capable of oxidizing the iron in Fe(II)-bleomycin to produce Fe(III). Also, superoxide and peroxide ($\cdot O_2^-$ and H_2O_2) are considered capable of reducing Fe(III) to Fe(II)[2] (Fong et al., 1976; Waters, 1948; Norman and Smith, 1965) and are thought to be present in solutions of Fe(II) undergoing autoxidation (Goto et al., 1970). One interpretation of the effect of superoxide dismutase and catalase on the degradation of DNA by bleomycin in the presence of xanthine oxidase and hypoxanthine is that the enzymatically generated $\cdot O_2^-$ is necessary to provide a continuous source of Fe(II) from adventitious Fe, in much the same way as other reducing agents. Superoxide dismutase removes this source of reducing equivalents. The stimulation of the reaction by catalase might be due to the local generation of O_2 from H_2O_2 produced in its enzymatic reaction. In this situation, O_2 generation would not occur at the expense of $\cdot O_2^-$. Superoxide dismutase would produce O_2 and H_2O_2 but only at the expense of $\cdot O_2^-$. Alternatively, catalase may possess contaminating Fe(III) not susceptible to dialysis but capable of participating in the reaction. Despite the need for further study of the role of reduced oxygen species in the degradation of DNA by bleomycin under various conditions, we emphasize that this analysis must consider the presence and action of Fe in such systems.

The model of bleomycin action proposed in Figure 4 involves no necessary direct interaction between reductants and bleomycin. Rather, these compounds are thought to generate Fe(II) from Fe(III), in particular where Fe(II) has not been added to the reaction. This role is contrasted to the documented direct activation of other drug molecules by reducing agents prior to interaction with DNA. For example, mitomycins are known to require reduction prior to their alkylation of DNA (Szybalski, 1964). Streptonigrin in its reduced form has been suggested to autoxidize with production of reduced oxygen radical intermediates which may then act to break DNA (Cone et al., 1976). We recognize that bleomycin may be altered, as it interacts with Fe(II) and O_2 during the degradation of DNA.

No role for metal ions has been postulated in the binding of bleomycin to DNA, in contrast to antibiotics such as chromomycin, which have an absolute requirement for divalent metal ions in order to bind to DNA (Ward et al., 1965). A recent experiment has also implicated metal ion in the binding of streptonigrin to DNA (White, 1977). It is recognized, however, that the binding parameters of bleomycin to DNA in the presence of metal ions may differ from those obtained previously in their absence (Chien et al., 1977). It is also possible that complexation of metal ion and drug could take place prior to the binding of this complex to DNA.

It has been observed that Fe is a ubiquitous contaminant of biochemical materials. For example, Springgate et al. (1973) found that Fe copurified with *E. coli* DNA polymerase I, and this Fe was not removed after extensive dialysis with *o*-phenanthroline, whereas the Zn(II), which is necessary for the function of the enzyme, was removed by this treatment. Hochstein et al. (1964) attributed stimulation of lipid peroxidation by nucleoside phosphates to Fe which was a contaminant in these compounds. Subsequent studies of lipid peroxidation have suggested (Poyer and McKay, 1971) that Fe is required for these reactions to occur. Therefore, the participation of Fe in the degradation of DNA by bleomycin is highly plausible, even in those systems to which exogenous Fe has not been added. This possibility assumes greater import when the extremely small concentrations (about 10^{-9}–10^{-10} M) of full-length DNA molecules commonly monitored in DNA breakage experiments using sucrose gradient techniques (as in Figure 2) are considered in relation to the levels of Fe which are found in such reaction mixtures (Table IV).

Further evidence that Fe(II) is the common cofactor by which various reductants act with bleomycin to degrade DNA is found in the following paper of this issue (Sausville et al., 1978), where the products of the degradation of DNA by bleomycin and Fe(II) are examined in detail. These results reinforce the suggestion that bleomycin in all systems thus far examined acts as both a chelating agent and a DNA-binding molecule to effect the efficient oxidative degradation of DNA with Fe(II).

[2] Although the more recent reviews cited in the text indicate direct reduction of Fe(III) by H_2O_2 as described in Figure 4, Haber and Weiss (1934) present evidence that solutions of H_2O_2 reduce Fe(III) by way of HO_2^-, the peroxide anion. Although at equilibrium Fe(III) is present in excess over Fe(II) due to the rapid reaction of Fe(II) with H_2O_2, it remains clear that Fe(II) can be generated from Fe(III) in solutions containing H_2O_2. It is for this reason that H_2O_2 is considered a reducing agent in this paper.

Acknowledgment

The authors express gratitude to Drs. P. Aisen, J. Wittenberg, and M. Horwitz for their helpful advice concerning these experiments. We thank Ms. E. Chalas for assistance in the determinations of Fe.

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Properties and Products of the Degradation of DNA by Bleomycin and Iron(II)†

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ABSTRACT: Bleomycin and Fe(II) together act to cause the highly efficient degradation of adenovirus-2 DNA to acid-soluble products when the drug and metal ion are present in a one- to twofold excess of DNA. Where the bleomycin–DNA ratio is less than 1, breakage of DNA occurs to a greater extent than is seen with Fe(II) in the absence of bleomycin. Breakage of DNA occurs from pH 4.5 to greater than 10 and is not greatly dependent on temperature. The appearance of activity between pH 4 and 6 is related to the appearance of a 1:1 Fe(II)–bleomycin complex with λ_{max} 476 nm, ϵ_M (apparent) = 3.8×10^2, and pK = 5.2. The reaction proceeds to completion in less than 1 min at 37 °C and requires the presence of molecular oxygen. Increased ionic strength inhibits the reaction. Degradation of DNA by Fe(II)–bleomycin is not inhibited by superoxide dismutase or catalase at high bleomycin concentrations; however, at low concentrations of bleomycin inhibition by superoxide dismutase, but not by catalase, is observed. All four bases in DNA are released by Fe(II)–bleomycin (T > C > A > G). Discrete nuclease-resistant oligonucleotides of average length 7–10 residues are formed in the limit degradation of DNA by Fe(II)–bleomycin. Mononucleotides and inorganic phosphate are not produced. A product which resembles malondialdehyde is found and its formation is maximal at a 1:1 Fe(II) to bleomycin ratio in reactions containing excess DNA. The products of the degradation of DNA by Fe(II)–bleomycin are in accord with the proposal that Fe(II) is the ultimate cofactor of bleomycin in degrading DNA.

Bleomycin, an antibiotic which both chelates metal ions (Nunn, 1976) and binds to DNA (Chien et al., 1977), causes the degradation of isolated DNA in a reaction that requires reducing agents for most efficient DNA breakage. Previous experiments have demonstrated that in the presence of bleomycin and a wide variety of organic reducing agents DNA is degraded to acid-soluble oligonucleotides with the release of free bases and compounds containing aldehyde functions (Haidle et al., 1972; Müller et al., 1972; Kuo and Haidle, 1974). We have recently focused our attention on the mechanism by which reducing agents act with bleomycin to degrade DNA. Recent communications from this laboratory have

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‡ Medical Scientist Trainee of the United States Public Health Service (Grant 5T 32 GM 7288).
§ Recipient of an Irma T. Hirschl Career Scientist Award.

0006-2960/78/0417-2746$01.00/0