Effect of Deoxyribonucleic Acid on the Production of Reduced Oxygen by Bleomycin and Iron†

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ABSTRACT: The binding of bleomycin to DNA in the presence and absence of ferric iron was measured by fluorescence spectroscopy. In millimolar concentrations of tris(hydroxymethyl)aminomethane, pH 7.5, approximately 80% of the bleomycin binds to DNA. Ferric iron seems to have no significant effect on the binding of DNA to bleomycin. The induction of oxygen uptake by ferrous iron and bleomycin was monitored in the presence and absence of DNA. DNA has no effect on the rate of oxygen uptake. Therefore, the iron binding site and the DNA binding site appear to be independent of each other. Under conditions where 80% of the bleomycin is bound to DNA, the ferrous iron–bleomycin-induced reduction of oxygen follows Michaelis–Menten kinetics. Ferrous iron autoxidation produces ethylene from methional. The addition of bleomycin greatly increases ethylene production. DNA, under conditions where 80% of the bleomycin is bound to DNA, inhibits ethylene production. Since ethylene is a measure of hydroxyl radical production, we conclude that DNA is able to compete with methional for the hydroxyl radical. We postulate a mechanism for DNA double-strand breaks in which the bleomycin selectively binds to DNA and recurrently produces the hydroxyl radical at that site. The localized generation of many hydroxyl radicals as provided by the proposed oxidation–reduction cycle mechanism may cause multiple strand breaks taking place on both strands of the DNA duplex leading to double-strand breaks. Since catalase, but not superoxide dismutase, is able to inhibit ferrous iron–bleomycin-induced products of the hydroxyl radical, hydrogen peroxide, but not the superoxide radical, is the immediate precursor of the hydroxyl radical.

In 1966, Umezawa and co-workers (Umezawa et al., 1966a,b) discovered that bleomycin, a polypeptide with a molecular weight of approximately 1450, possessed antimicrobial and antitumor activity. The polypeptide, when isolated, consists of a group of glycopeptides that differ only in the terminal amine moiety. Bleomycin A_1 is the principal component of the mixture used clinically.

While the mechanism that is responsible for the antitumor activity is unknown, its principal biochemical activity appears to be its ability to bind and break DNA (Suzuki et al., 1969; Takeshita et al., 1974, 1976; Iqbal et al., 1976; Kohn & Ewig, 1976; Haidle, 1971; Haidle et al., 1972; Kuo et al., 1973; Bearden & Haidle, 1975; Bearden et al., 1977; Lloyd et al., 1978; Sausville et al., 1978a,b). Kohn has suggested that bleomycin-induced DNA degradation may be associated with cell toxicity (Kohn & Ewig, 1976).

Bleomycin appears to break DNA efficiently only in the presence of oxygen, ferrous iron, and reducing agents such as mercaptoethanol, ascorbate, and dithiothreitol (Sausville et al., 1976, 1978a,b; Lown & Sim, 1977; Suzuki et al., 1969; Onishi et al., 1975). The DNA is degraded to acid-soluble oligonucleotides and free bases (Haidle et al., 1972; Müller et al., 1972; Kuo & Haidle, 1974).

It appears that ferrous iron and bleomycin form a complex which is able to reduce molecular oxygen to the superoxide radical, hydrogen peroxide, and the hydroxyl radical. The byproduct is a ferric iron–bleomycin complex. The increase in efficiency of DNA strand breakage due to the addition of reducing agents is probably caused by their ability to reduce ferric iron back to the ferrous state (Sausville et al., 1978a,b; Caspary et al., 1979, 1981).

We have demonstrated that the ferrous iron–bleomycin complex in the absence of DNA reduces oxygen and follows Michaelis–Menten kinetics (Caspary et al., 1979). This implies a cyclic mechanism for oxygen reduction in which oxygen oxidizes the ferrous iron–bleomycin complex to the ferric state (Figure 1) and generally agrees with a proposal by Sausville et al. (1978a). The ferric iron can be either reduced by cellular reducing agents or replaced by ferrous iron to continue the cycle (Caspary et al., 1981). A number of intermediates in the cycle have been identified (Caspary et al., 1981; Burger et al., 1979a,b; Sausville et al, 1976, 1978a,b; Sugiura & Kikuchi, 1978).

Our objectives in this study are to determine whether this reaction follows Michaelis–Menten kinetics when bleomycin is bound to DNA and whether the hydroxyl radical is one of the products of this reaction.

Materials and Methods

Bleomycin–DNA Binding. We followed the method described by Chien et al. (1977) to measure the binding of bleomycin to DNA. Increasing amounts of bleomycin alone or with ferric iron (1:1 mole ratio) were added to 0.125 mM calf thymus DNA in 1 mM tris(hydroxymethyl)aminomethane (Tris), pH 8.0. The fluorescence was measured at 355 nm after excitation at 300 nm on an Aminco Bowman fluorescence spectrophotometer. The addition of an equimolar concentration of ferric iron to bleomycin had no effect on the fluorescence spectrum of bleomycin. DNA quenched the fluorescence of bleomycin in both the presence and absence of ferric iron. Measurement of DNA quenching with increasing concentrations of bleomycin in the presence and absence of ferric iron made it possible to calculate the association constant, the number of bleomycin molecules bound per DNA nucleotide, and the ratio of moles of bleomycin to moles of DNA required to obtain a solution with 80% of the bleomycin bound. These quantities were calculated from the equation (Chien et al., 1977)

$$\frac{1}{C} = \frac{1}{nKD_0B} + \frac{1}{nD_0}$$

where B represents the concentration of free bleomycin, D_0

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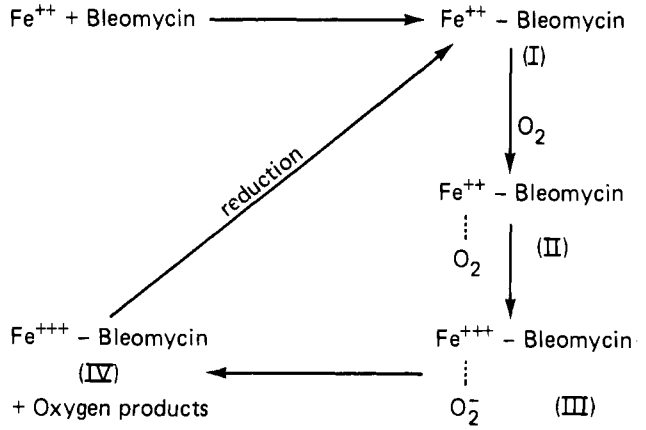

FIGURE 1: Proposed mechanism for the reaction of bleomycin, iron, and oxygen. Structures II and III are included to demonstrate a dynamic process whereby an electron is transferred from the ferrous iron to oxygen. The actual structure and binding characteristics of these species have not been determined (Caspary et al., 1981).

the concentration of DNA added, C the concentration of bleomycin bound to DNA, and n the number of bleomycin molecules bound per DNA nucleotide.

Oxygen Reduction. The oxygen content of the reaction mixtures was measured with a Clark electrode in a Yellow Springs Model 53 instrument. Reaction mixtures (final volume 1 mL) contained 1 mM Tris buffer (pH 7.6), 10^{-5} M bleomycin A_2, 7×10^{-5} M calf thymus DNA, and increasing concentrations of ferrous sulfate. Buffer and water were aerated by bubbling with air for 3 min at 37 °C. Bleomycin was added to the reaction vessel, the electrode was inserted, and the system was equilibrated for 1 min while being monitored for oxygen content. $FeSO_4$ (50 μL) (10 mM) was injected into the reaction vessel, and the change in oxygen content was determined. $FeSO_4$ solutions were made fresh daily in water that had been sparged with N_2. Rates of oxygen consumption were calculated from the initial, linear electrode response. The value used for the dissolved oxygen content was 0.16 mM (Chappell, 1964).

Ethylene Formation. Beauchamp & Fridovich (1970) described a gas chromatographic assay for the hydroxyl radical. Adapting this technique, we placed 20 μL of methional and 5 mL of 1 mM Tris buffer, pH 7.5, into a rubber-stoppered flask. Catalase (20 μg/mL) or superoxide dismutase (100 μg/mL) was added when appropriate. The flask was shaken at 37 °C in a water bath. Ferrous sulfate (final concentration 5×10^{-4} M) was injected into the flask to initiate the reaction. As appropriate intervals, 9 mL of the gas was drawn into a Hamilton gas-tight syringe and injected into a Shimadzu GC-6AM gas chromatograph fitted with 2.5 m × 3 mm Porapak type Q (100–120 mesh) column. The column temperature was maintained at 50 °C when not in use to prevent water absorption. Absorption curves were recorded on a 7132A Hewlett Packard strip-chart recorder. The areas under the absorption curves were cut out and weighed. Concentrations were obtained by comparing the weights of the paper under the absorption curves from a known amount of ethylene.

Results

By measuring the quenching of bleomycin fluorescence by DNA, we calculate that, in 1 mM Tris, pH 8.0, approximately 1 mol of bleomycin is bound to 10 mol of DNA nucleotides in either the presence of the absence of ferric iron (Figure 2). The equilibrium constant describing the binding of bleomycin to DNA is 4.1×10^5 M^{-1}. In the presence of ferric iron, the value is 9.6×10^5 M^{-1}. We calculate that, at a concentration

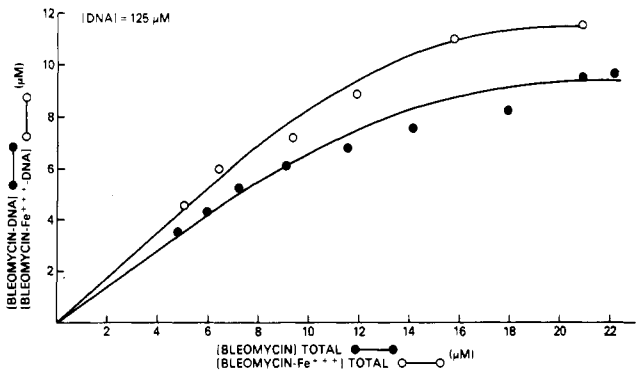

FIGURE 2: Binding of bleomycin to DNA in the presence and absence of iron salts. The final reaction mixture contains 0.125 mM calf thymus DNA and 1 mM Tris, pH 8.0. Increasing amounts of bleomycin (●) or an equimolar solution or an equimolar solution of ferric iron–bleomycin (○) was added, and the fluorescence emission was measured at 355 nm after excitation at 300 nm. The graph shows saturation of the binding site with increasing concentrations of bleomycin (●) of ferric iron–bleomycin (○).

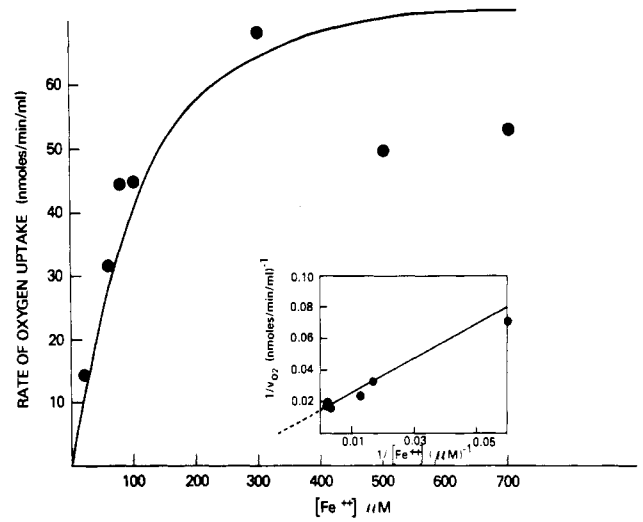

FIGURE 3: Michaelis–Menten plot of oxygen consumption vs. ferrous iron concentration. The insert is a Lineweaver–Burk plot of the data. The protocol is described under Materials and Methods. The reaction mixtures contain 7×10^{-5} M DNA in 1 mM Tris, pH 7.6, with increasing amounts of ferrous iron with and without 10^{-5} M bleomycin. The initial reaction rates of oxygen induced by bleomycin and ferrous iron were calculated as the difference between the rates in the presence and absence of bleomycin.

of DNA 7-fold greater than the concentration of the bleomycin, approximately 80% of the bleomycin is dynamically bound to DNA in both the presence and or absence of ferric iron. The strength of the binding is a function of the buffer. For example, in 0.01 M maleate, pH 6.2, a DNA to bleomycin ratio of 200 to 1 is required to ensure that 50% of the bleomycin is bound.

At a ferrous iron concentration of 5×10^{-4} M in 0.1 M Tris, pH 7.5, the rate of oxygen consumption increased linearly with increasing concentrations of a 7:1 DNA:bleomycin mixture. When increasing concentrations of ferrous iron were added to reaction mixtures containing 10^{-5} M bleomycin A_2, 7×10^{-5} M DNA, and 1 mM Tris at pH 7.5, the initial rates of oxygen consumption increased until saturation was reached. The experimental data followed Michaelis–Menten kinetics (Figure 3). An analysis of these data by a Lineweaver–Burk plot (Figure 3) yielded $K_m = 80.5$ μM and $V_{max} = 73$ nmol of O_2 min^{-1} mL^{-1}. The specific activity of bleomycin A_2 in the presence of a 7-fold excess of DNA at 1 mM Tris, pH 7.5, is 7300 μmol of O_2 consumed min^{-1} (μmol of bleomycin A_2)$^{-1}$.

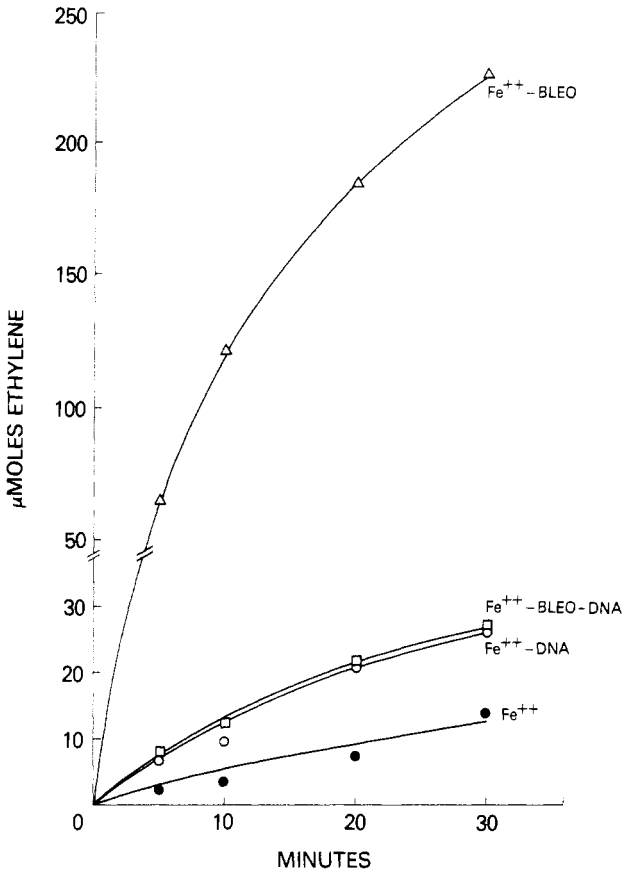

FIGURE 4: Production of ethylene from bleomycin and ferrous iron in the presence and absence of DNA. The final reaction mixture contains 5×10^{-4} M ferrous iron, 20 μL of methonial in 5 mL of 1 mM Tris, pH 7.5, 5.6×10^{-5} M bleomycin, when used, and 4×10^{-4} M calf thymus DNA, when used. Ethylene is measured quantitatively on a gas chromatograph with a flame ionization detector.

The rate of oxygen uptake induced by an equimolar solution of bleomycin and ferrous iron in 1 mM Tris, pH 8.0, is not affected by a 7-fold excess of DNA where 80% of the bleomycin is bound to DNA (data not shown).

Since ferrous iron causes the univalent reduction of oxygen (Weiss, 1935, 1953), the reduction of oxygen by ferrous iron and bleomycin is expected to produce the superoxide radical, hydrogen peroxide, and ultimately the hydroxyl radical. Ethylene formation from methional (Beauchamp & Fridovich, 1970) is a measure of the production of the hydroxyl radical if quenched in the presence of a radical scavenger like ethanol. Incubation of ferrous iron in an aerobic Tris-buffered solution at pH 7.5 with methional resulted in a gas chromatographic peak with characteristics similar to that of pure ethylene. Cochromatographing a standard sample of ethylene with the gaseous products of ferrous iron autoxidation and methional produced one peak. The omission of ferrous iron, methional, or oxygen eliminated the production of ethylene. When 5 and 10% ethanol (a hydroxyl radical scavenger) was added to a solution containing 5×10^{-4} M calf thymus DNA, 5×10^{-4} M ferrous iron, and 5.6×10^{-5} M bleomycin in 1 mM Tris, ethylene production was inhibited by 55 and 66%, respectively.

The addition of 5.6×10^{-5} M bleomycin A_2 to 5×10^{-4} M ferrous iron markedly increased the production of ethylene (Figure 4). The addition of 5×10^{-4} M DNA to ferrous iron in the absence of bleomycin had no significant effect on the rate of ethylene production induced by ferrous iron. However, 5×10^{-4} M DNA greatly reduced the concentrations of ethylene induced by ferrous iron and bleomycin. Although we found that catalase had an inhibitory effect on ethylene production in the presence of bleomycin, we were unable to unequivocally attribute the inhibition to the bleomycin-induced mechanism (Table I). The inhibition could be due to a quenching of the hyroxyl radical formed by the spontaneous ferrous iron autoxidation. To show that catalase inhibited the bleomycin-induced product of ethylene, we adjusted the conditions to 50 mM Tris since increasing the Tris concentration increases DNA binding (Chien et al., 1977). At this buffer concentration, the induced formation of ethylene was negligible so we doubled the bleomycin concentration to 1.2×10^{-4} M. Under these conditions, catalase reduces the concentration of ethylene produced by ferrous iron, bleomycin, and DNA by 10 μmol. This was larger than the amount of ethylene produced in the presence of just ferrous iron and buffer (6 μmol of ethylene). Thus, at least part of the inhibition observed in the presence of bleomycin is due to a bleomycin-induced mechanism. Superoxide dismutase had no effect on ethylene production under any of the conditions tested.

Table I: Effect of Buffer Concentrations on Ethylene Production[a]

complex	compd added	ethylene (μmol) expt A	expt B
Fe^{2+}		40	7
Fe^{2+}	catalase	14	1
Fe^{2+}	catalase (inact)	41	8
Fe^{2+}	SOD	39	7
Fe^{2+}	SOD (inact)	39	7
Fe^{2+}-Bleo		87	18
Fe^{2+}-Bleo	catalase	46	9
Fe^{2+}-Bleo	catalase (inact)		20
Fe^{2+}-Bleo	SOD		17
Fe^{2+}-Bleo	SOD (inact)		17
Fe^{2+}-DNA		37	5
Fe^{2+}-DNA	catalase	16	1
Fe^{2+}-DNA	catalase (inact)	35	6
Fe^{2+}-Bleo-DNA		62	13
Fe^{2+}-Bleo-DNA	catalase	43	3
Fe^{2+}-Bleo-DNA	catalase (inact)	60	
Fe^{2+}-Bleo-DNA	SOD	64	14
Fe^{2+}-Bleo-DNA	SOD (inact)	63	13
Fe^{2+}-Bleo-DNA	ethanol (5%)	28	
Fe^{2+}-Bleo-DNA	ethanol (10%)	21	

[a] The final reaction mixtures contained 5×10^{-4} M ferrous iron, 5.6×10^{-5} M (experiment A) or 1.2×10^{-4} M (experiment B) bleomycin (Bleo) when used, 4×10^{-4} M calf thymus DNA when used, and 1 mM Tris, pH 7.5 (experiment A), or 50 mM Tris, pH 7.5 (experiment B). Ethylene was measured quantitatively on a gas chromatograph (see Materials and Methods). Inactive SOD and catalase were prepared by boiling for 10 min.

Discussion

The pharmacological action of bleomycin may be due to its interaction with ferrous iron and its resultant ability to cause DNA strand breaks (Sausville et al., 1976, 1978a,b; Lown & Sim, 1977). We have recently shown that the reduction of oxygen by bleomycin in the presence of ferrous iron follows classical Michaelis–Menten kinetics. This implies a cyclic oxidation–reduction cycle (Figure 1). The oxidation phase is driven by oxygen (Caspary et al., 1979). Celluar reducing agents may drive the reduction phase in vivo (Caspary et al., 1981). Since bleomycin binds to DNA, it seemed critical to explore the effect of DNA on oxygen reduction.

While examining the binding of bleomycin to DNA, we discovered that the magnitude of this binding is a function of such factors as buffer, pH, and ionic strength. The optimum conditions for measuring ferrous iron–bleomycin-induced oxygen reduction and for determining maximum binding of bleomycin to DNA are not identical. Oxygen reduction is best

measured in 0.1 M maleate, pH 6.2, because the spontaneous, ferrous iron induced autoxidation is inhibited under these conditions. This permitted us, in a previous report (Caspary et al., 1979), to quantify oxygen reduction induced by ferrous iron with bleomycin since only an insignificant spontaneous rate of oxygen reduction had to be subtracted from the rates observed in the presence of bleomycin. However, under those conditions, bleomycin did not bind well to DNA. Since the purpose of the experiments reported here is to determine the effect of DNA on oxygen reduction by bleomycin and ferrous iron, we repeated the oxygen uptake studies under conditions designed to optimize DNA binding at the expense of completely inhibiting the spontaneous autoxidation of ferrous iron. Thus, all the work reported here was performed in Tris buffer.

In the presence of 1 mM Tris, pH 8.0, 10^{-5} M bleomycin, and 7×10^{-5} M DNA, approximately 80% is bound to DNA, and the ferrous iron–bleomycin reduction of oxygen follows classical Michaelis–Menten kinetics (Figure 3). We conclude that the oxidation–reduction cycle that we proposed in the absence of DNA (Caspary et al., 1979) is effective when bleomycin is bound to DNA (Figure 1).

Our data show that ferric iron had little effect on the association constants describing the binding of bleomycin A_2 to DNA (Figure 2). Since DNA has no effect on the rates of oxygen reduction induced by the ferrous iron–bleomycin complex, we infer that the DNA binding site and the metal binding site of bleomycin A_2 are essentially independent of each other. This conclusion is not inconsistent with the molecular structure of bleomycin. The DNA binding site is located on the tripeptide S chain (Chien et al., 1977). The terminal amine moiety, the pyrimidine, and perhaps the imidazole groups have been implicated as the metal binding site (Gupta et al., 1979; Dabrowiak et al., 1978a,b). In view of the considerable distance between these two sites and the restricted flexibility of the bleomycin chain, it is clearly plausible that the activities of these two sites would be essentially independent.

Bleomycin enhances ethylene formation 20-fold over the ferrous iron induced control. Thus, the reaction of the ferrous iron–bleomycin complex produces the hydroxyl radical. The addition of DNA to the ferrous iron–bleomycin complex substantially reduces the ethylene concentrations under conditions where substantial amounts of DNA are bound to bleomycin (Figure 4).

Two explanations are possible for this observed reduction of ethylene concentrations. Either DNA inhibits the formation of or reacts with the hydroxyl radical. Since DNA has no effect on ferrous iron autoxidation and since the dismutation of the superoxide radical to form hydrogen peroxide and the hydroxyl radical is extremely rapid at pH 8.0 (Behar et al., 1970), the quenching of ethylene that is observed in the presence of DNA is probably not due to an inhibition of the production of ethylene. Since the reaction between nucleoside moieties and the hydroxyl radical is essentially diffusion controlled [$k = 10^9$–10^{10} M^{-1} s^{-1} (Anbar & Neta, 1967)], we would expect that DNA would effectively compete with methional for the hydroxyl radical. In the presence of bleomycin, the hydroxyl radical may be produced adjacent to the DNA duplex due to the binding of bleomycin to DNA, and consequently, the DNA effectively competes with methional for the hydroxyl radical.

This explanation supports the suggestion that the hydroxyl radical is responsible for the bleomycin-induced strand breaks (Lown & Sim, 1977; Sugiura, 1979; Ishida & Takahashi, 1975; Burger et al., 1979a,b) though it does not rule out a role in DNA strand breakage for other reduced oxygen species. In 1972, Haidle et al. observed the formation of DNA bases when DNA was incubated with bleomycin and ferrous iron. Although Haidle and co-workers did not suggest a hydroxyl radical mediated reaction, Rhaese & Freese (1968) suggested a mechanism for the formation of DNA bases due to the reaction between DNA and the hydroxyl radicals.

Various investigators have shown that ferrous iron produces random, nonspecific strand breaks in DNA (Lown & Sim, 1977; Takeshita et al., 1976; Haidle et al., 1972; Povirk et al., 1977; Suzuki et al., 1969) presumably due to a site-nonspecific autoxidation of ferrous iron and subsequent random hydroxyl radical attack on DNA. The addition of bleomycin to a mixture of ferrous iron and bleomycin promotes cleavage at the G-C and C-T sequences in DNA (Takeshita et al., 1976). This has been attributed to a specific binding of bleomycin to certain sequences of DNA.

The number of double-strand breaks formed in the presence of ferrous iron and bleomycin appears to be greater than the number expected from a random coincidence of single-strand breaks (Pivirk et al., 1977). Lloyd et al. (1978) proposed that bleomycin forms dimers in which chemically reactive groups bind and break the complimentary strands.

We would propose that the high incidence of double-strand breaks might be explained by the selective binding of bleomycin to certain DNA sequences and the recurrent production of the hydroxyl radical. The localized generation of many hydroxyl radicals as provided by the proposed oxidation–reduction cycle mechanism allows for multiple single-strand breaks taking place on both strands of a DNA duplex. These multiple strand breaks could cause double-strand breaks around the binding site of a single bleomycin molecule.

Sausville et al. (1978a) proposed a model that a ternary complex among iron, bleomycin, and oxygen is formed in which the ferrous iron is oxidized to the ferric state with the concomitant reduction of the reduced oxygen species. They suggest that the superoxide radical may cause the reduction of ferric iron to the ferrous state. The two models are in basic agreement though we have reported that the superoxide radical is unable to reduce the ferric iron–bleomycin complex (Caspary et al., 1981).

Burger et al. (1979a,b) identified a ternary intermediate among iron, bleomycin, and oxygen and identified it as a ferrous iron–bleomycin–oxygen complex. We have observed a ferric iron–bleomycin–reduced oxygen complex at pH 9.3 (Caspary et al., 1981). However, the observations of Burger et al. were made under different experimental conditions. We have found that this system is extremely sensitive to buffer and pH effects. The two observations may well be monitoring different stages of the ternary complex during its migration from the ferrous to the ferric state (Figure 1). The stability of these intermediates changes with pH and buffer. In any event, the oxidation–reduction cycle of the iron–bleomycin–oxygen complex appears reasonable in accounting for the breakage of DNA in biochemical systems.

According to our data, catalase, but not superoxide dismutase, is able to inhibit ethylene formation due to the reduction of oxygen by the ferrous iron–bleomycin complex. Under the original conditions used (Table I, experiment A), there is the potential that significant autoxidation of ferrous iron may occur; thus, the inhibition that is observed may not be due to the ferrous iron–bleomycin-induced production of ethylene. In order to show unequivocally that catalase inhibits ethylene produced by the ferrous iron–bleomycin complex, we had to modify conditions under which catalase inhibition of

ethylene formation was monitored.

Catalase inhibits ethylene formation both in the preesence and absence of bleomycin and in the presence and absence of DNA. However, in 1 mM Tris where 80% of bleomycin is bound to DNA, the decrease in the amount of ethylene might be due to an inhibition of the ethylene formed from the spontaneous autoxidation of ferrous iron. At 50 mM Tris, the inhibition of ethylene formation by catalase in the presence of bleomycin cannot be totally explained by the inhibition of the ethylene produced by ferrous iron autoxidation even if the bleomycin-induced reaction has no effect on the spontaneous ferrous iron autoxidation. Thus, catalase is able to inhibit the ethylene produced by H_2O_2 which was formed by the bleomycin-induced reduction of oxygen. Superoxide dismutase (SOD) has no effect on ethylene production either in the presence or absence of DNA or in the presence or absence of bleomycin. Thus, the superoxide radical, while it may be a precursor to the hydroxyl radical, is not directly involved in its production. This agrees with the conclusion that the Haber–Weiss reaction cannot explain the production of the hydroxyl radical (Czapski & Allen, 1972; McCord & Day, 1978; Halliwell, 1976; Bielski & Allen, 1977).

This experiment also indicated that H_2O_2 is a direct precursor of and obligate intermediate in the production of the hydroxyl radical. Since $O_2^{-}\cdot$ is not involved in the rate-determining step, the production of the hydroxyl radical probably involves a Fenton-like reaction:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH\cdot$$

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