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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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provided strong evidence that organic reducing agents allow the continual generation of Fe(II) from Fe(III) which contaminates reaction mixtures. Fe(II) may then form a complex with bleomycin while bound to DNA. It is proposed that the oxidation of the Fe(II)–bleomycin–DNA complex results in DNA damage (Sausville et al., 1976, 1978).

If this model is correct, it is necessary to demonstrate that the properties and products of DNA degradation by bleomycin and added Fe(II), but in the absence of organic reducing agents, are related to the properties and products of DNA degradation which have been observed with organic reducing agents but in the absence of added Fe(II). The experiments in this paper describe in considerable detail the products and properties of the degradation of DNA by bleomycin and Fe(II).

Materials and Methods

Enzymes and Reagents. $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ and $Fe(NH_4)(SO_4)_2 \cdot 12H_2O$ (Mallinkrodt analytical reagents) were sources of Fe(II) and Fe(III), respectively. These were dissolved in deionized H_2O immediately prior to use. Tris,[1] Hepes, 2-thiobarbituric acid, adenine, thymine, guanine, cytosine, and bovine serum albumin (fraction V) were from Sigma; $Na(C_2H_3O_2)$, glycine, and other buffer components were from Fisher; pancreatic DNase (EC 3.1.4.5), spleen exonuclease (EC 3.1.4.18), venom exonuclease (EC 3.1.4.1), bacterial alkaline phosphatase (BALP; EC 3.1.3.1), and calf thymus DNA were from Worthington; [^{3}H]thymidine (40–60 Ci/mmol) and $^{32}P_i$ (carrier free) were from ICN. Methanol for high pressure liquid chromatography was from Burdick and Jackson. Bleomycin and other chemicals were as described previously (Sausville et al., 1978).

DNA. The preparation of radioactively labeled adenovirus-2 DNA was from the virion, as described by Horwitz (1971). Where $^{32}P_i$ was to be incorporated into viral DNA, infection of KB cells was carried out as described, except that at 7 h after infection the medium was replaced by one containing $1/20$ the usual concentration of unlabeled phosphate and 60 μCi/mL $^{32}P_i$ and 0.5 μCi/mL [^{3}H]thymidine. An identical aliquot of [^{3}H]thymidine was added at 18 h after infection. The radioactively labeled viral DNA was dialyzed against 2 mM Tris-HCl, pH 7.5, and its concentration was estimated by assuming a molar extinction coefficient of 6.6×10^3 M^{-1} at 260 nm.

Analysis of Damage to DNA. The production of acid-soluble radioactivity from labeled DNA after reaction with bleomycin and Fe(II) was followed. In general, reaction mixtures were prepared at 2–4 °C with all components except Fe(II). After equilibration at 37 °C, Fe(II) was added and incubation continued for 15 min. EDTA and bovine serum albumin were added to final concentrations of 0.05 M and 1 mg/mL, respectively. Perchloric acid was added to a concentration of 0.5 M and samples were separated into acid-soluble and acid-insoluble fractions by centrifugation at 6000*g* for 20–30 min. The radioactivity in an aliquot of the acid-soluble fraction was determined in 8 mL of Yorktown TT 21 scintillant except where noted. To obtain good reproducibility (±15%) in these assays, reaction tubes were cleaned in chromic acid–sulfuric acid followed by thorough rinsing with distilled H_2O. They were then washed with Haemo-Sol (Haemo-Sol, Inc., Baltimore) detergent followed by subsequent vigorous rinsing with distilled H_2O. Alkaline sucrose density gradient analysis of labeled DNA after reaction with bleomycin and Fe(II) was conducted as described previously (Sausville et al., 1978), using the modifications noted in the figure legends.

Fractionation of Oligonucleotides. The oligonucleotides present in a limit digest of adenovirus-2 DNA after reaction with bleomycin and Fe(II) were analyzed by DEAE-cellulose chromatography (Whatman DE-52) in 7 M urea (Tomlinson and Tener, 1963), as described by Friedman and Smith (1972). Marker oligonucleotides for this separation were prepared by digesting 4.3×10^{-3} M calf thymus DNA with 20 μg/mL pancreatic DNase for 180 min at 37 °C in 0.05 M Tris-HCl, pH 7.2, containing 0.05 M $MgCl_2$. The mixture was heated at 95 °C for 7 min to inactivate the nuclease. Labeled oligonucleotides in column fractions were determined by adding 16 mL of Yorktown TT 21 scintillant to 2 mL of column fraction. After elution from the column, nucleotides were desalted as described by Rushizky and Sober (1962) and redissolved in H_2O. The susceptibility of these fractions to sequential nuclease treatment was carried out as described in the table legends. After nuclease treatment, the presence of ^{32}P in a form sensitive to BALP was assayed by following the release of label to a form not adsorbed to Norit 211 FQP (Eastman) essentially as described by Wright et al. (1971).

Separation of Bases. Resolution of the four bases released from calf thymus DNA after reaction with bleomycin and Fe(II) was achieved using a Partisil PXS 10/25 ODS-2 microparticle column (25 cm × 4.6 mm, Whatman) attached to a Model 100 high-pressure liquid chromatography pump (Altex). Aliquots of reaction mixtures were injected with a Rheodyne (20 μL) looped injector. The absorbance at 254 nm of the column eluate was recorded (0.005 full scale) using an Altex Model 153 analytical UV detector and Model 210 recorder. The amount of base present was determined by peak weight in comparison to a standard curve obtained under identical conditions to the reaction mixture. The integrity of the standard curve was examined before each determination.

Adenine was separated using an eluant containing 20 mM KH_2PO_4, 12.5% methanol, with sufficient concentrated HCl added to give pH 3.70. At a flow rate of 2 mL/min, the retention time was 7.2 min. Cytosine, guanine, and thymine were separated from the other reaction components in an eluant containing 20 mM KH_2PO_4 and HCl to give a pH of 3.35. At a flow rate of 3 mL/min, retention time for these bases was 2, 8.5, or 11.5 min, respectively.

Generation of a Malondialdehyde-like Product. Aldehydes react with 2-thiobarbituric acid to form colored products (Waravdekar and Saslow, 1959; Schmidt, 1959). Malondialdehyde yields a characteristic chromophore with λ_{max} 532 nm. The assay employed here for the production of a malondialdehyde-like substance after reaction of calf thymus DNA with bleomycin and Fe(II) is as follows. An aliquot (0.2–0.5 mL) from reaction mixtures described in the figure legends was diluted to 2 or 2.2 mL with 0.6% 2-thiobarbituric acid in H_2O. The mixture was heated for 20 min in a capped test tube in a boiling H_2O bath. After cooling for 5 min at room temperature, absorbance at 532 nm was read against a reference solution which received H_2O instead of an aliquot of reaction mixture. Under these conditions, authentic malondialdehyde generated by hydrolysis of 1,1,3,3-tetramethoxypropane (Aldrich) as described by Kwon and Watts (1963) gave a chromophore which had ϵ_M (apparent) = 1.6×10^5 M^{-1}. Fe(II) or Fe(III), bleomycin, DNA, and phosphate buffers in the concentrations used did not interfere with the determination of malondialdehyde-like substances by this method.

[1] Abbreviations used are: BALP, bacterial alkaline phosphatase; Tris, tris(hydroxymethyl)aminomethane; P_i, inorganic phosphate; DEAE, diethylaminoethyl; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; EDTA, (ethylenedinitrilo)tetraacetic acid.

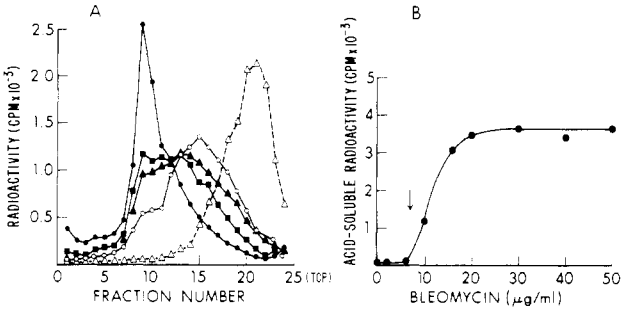

FIGURE 1: Degradation of DNA by bleomycin and Fe(II). (A) Reactions contained in a total volume of 100 μL: 22 μM adenovirus [^{3}H]DNA, 0.05 M phosphate buffer, pH 7.0, and the following additions. None or 10 μg/mL bleomycin (●); 20 μM Fe(II) (■); 20 μM Fe(II) plus 5 μg/mL bleomycin (▲); 20 μM Fe(II) plus 10 μg/mL bleomycin (○); 20 μM Fe(II) plus 15 μg/mL bleomycin (△). A portion of the reaction mixture was layered on an alkaline sucrose gradient. (B) Reaction mixtures contained in a final volume of 50 μL: 4.9 μM adenovirus [^{3}H]DNA (3500 cpm), 0.05 M phosphate buffer, pH 7.0, 1×10^{-4} M Fe(II), and the indicated concentration of bleomycin. The arrow indicates the concentration of bleomycin approximately equimolar with added DNA. Acid-soluble radioactivity was measured.

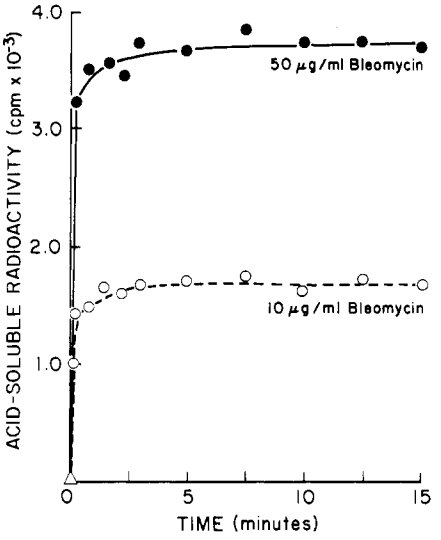

FIGURE 2: Kinetics of the degradation of DNA by Fe(II) and bleomycin. Reaction mixtures contained 4.9 μM of adenovirus [^{3}H]DNA (3600 cpm), 1×10^{-4} M Fe(II), and bleomycin at the indicated concentrations. At $t = 0$, Fe(II) was added, and, at the indicated times, aliquots of the reaction mixture were made 0.05 M in EDTA to terminate the reaction. Acid-soluble radioactivity was determined.

Optical Spectroscopy. Visible spectra of Fe(II)–bleomycin under various conditions were recorded with a Cary 14R recording spectrophotometer using optical cells with a 1-cm light path. A solution (3 mL) of bleomycin in the main compartment of a thunberg cuvette was deoxygenated by vigorously flushing with H_2O-saturated ultrapure argon (Linde) for 20 min at ambient temperature with continuous gentle agitation. Subsequently, anaerobically prepared aqueous dithionite was added through the rubber septum of the thunberg sidearm using a syringe (Hamilton) to give a final concentration of about $0.5\text{–}1 \times 10^{-2}$ M. In the titration of bleomycin with Fe(II), aliquots of the metal ion were then transferred from a stock deoxygenated aqueous solution of the metal ion through the septum. Otherwise, the solution of Fe(II) was deoxygenated in the sidearm prior to the addition of dithionite and subsequently mixed with the contents of the main compartment. The reference solution was H_2O. For studies of the pH dependence of Fe(II)–bleomycin formation, buffer solutions were prepared using a Radiometer pH meter 26. Absorbances of Fe(II)–bleomycin in buffers of differing pH were fitted by least-squares analysis for determination of pK as described by Peisach and Mannering (1975).

Results

Characteristics of the Degradation of DNA by Fe(II) and Bleomycin

Dependence on Fe(II) and Bleomycin. Fe(II) and bleomycin act to degrade DNA to a greater extent than either acting alone (Figure 1A). In these experiments the molar ratio of bleomycin and DNA is less than unity, whereas Fe(II) is approximately equimolar to DNA and in excess of bleomycin. Acid-soluble DNA degradation products are not observed. At 1–2 mol of bleomycin/mol of DNA nucleotide, and in the presence of excess Fe(II), DNA degradation proceeds completely to acid-soluble products (Figure 1B). It can be observed from Figure 1B that in the absence of bleomycin no detectable acid-soluble degradation products are formed. Thus, although Fe(II) produces breaks in DNA, highly efficient degradation of DNA in this system requires both Fe(II) and bleomycin. When bleomycin is present in excess of the molar concentration of DNA nucleotides, acid-soluble degradation products do occur at less than equivalent concentrations of Fe(II) in relation to bleomycin, but not at less than Fe(II)/bleomycin = 0.1 (data not shown).

Since the assays of Figure 1 measure the amount of DNA degradation after a 15-min incubation, the kinetic behavior of the reaction of bleomycin and Fe(II) with DNA is of importance and is examined in Figure 2. The degradation of DNA seen in the presence of bleomycin and Fe(II) reaches a plateau in less than 1 min when bleomycin is present at either 50 or 10 μg/mL. When additional Fe(II) is added to the reaction containing 10 μg/mL bleomycin after it had already reacted with excess Fe(II), no additional degradation is seen even if more bleomycin is added with the readdition of Fe(II) (data not shown). These results imply that the products of the degradation of DNA by bleomycin and Fe(II) formed at 10 μg/mL bleomycin are possibly refractory to the further action of bleomycin and Fe(II). Alternatively, the accumulation of Fe(III) in this system after oxidation of Fe(II) could potentially interfere with the action of added Fe(II) and bleomycin (Sausville et al., 1978).

Effects of pH and Buffer Composition

It may be seen in Figure 3A that the degradation of DNA by Fe(II) and bleomycin is remarkably dependent on the nature of the buffer used in the reaction, with a less evident dependence on pH. For example, as measured by this assay, phosphate buffer allows a greater amount of damage to DNA than is expressed in Tris-HCl at pH 7.2 or in acetate at pH 6.0. A smaller but very reproducible difference is observed between Tris-HCl and Gly-OH buffers at pH 9.1. Completely analogous results were obtained in reactions not shown which were done with SV40 DNA instead of adenovirus DNA. It is apparent that the DNA is subject to appreciable damage from Fe(II) and bleomycin over a wide pH range (pH 4.5–10).

To ascertain whether differences in activity are a function of kinetic properties of the reaction in different buffers, the experiments of Figure 3B were performed. For reasons not clear at the present time, in Tris-HCl or acetate buffers, there is a characteristic maximum in the amount of acid-soluble degradation products produced at very early times after initiating the reaction with a subsequent slow decline. This phenomenon is not an artifact of liquid scintillation counting, since

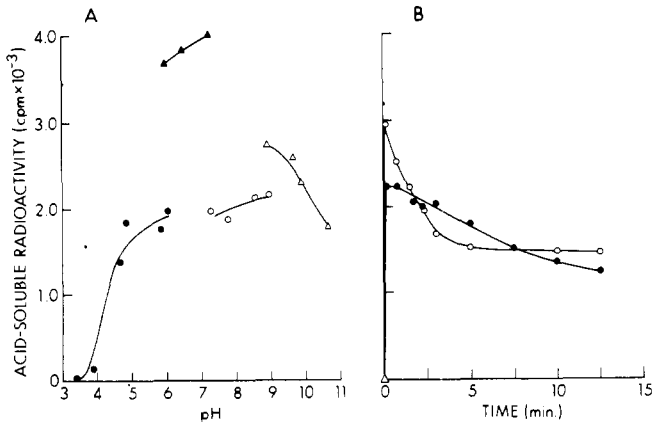

FIGURE 3: Effect of pH and buffer composition on the degradation of DNA by Fe(II) and bleomycin. (A) Reaction mixtures contained 4.0 μM adenovirus [^{3}H]DNA (3800 cpm), 50 μg/mL bleomycin, and 1 × 10^{-4} M Fe(II) in a buffer which was 0.05 M in either acetate (●), phosphate (▲), Tris-HCl (○), or glycine-NaOH (△). Acid-soluble radioactivity was determined. (B) Kinetics of the degradation of DNA by Fe(II) and bleomycin in 0.05 M Tris-HCl, pH 7.2 (○), or 0.05 M acetate, pH 6.0 (●). Reactions were carried out as described for Figure 2, except that phosphate buffer was replaced as indicated. A sample in either buffer prior to addition of Fe(II) is shown (△).

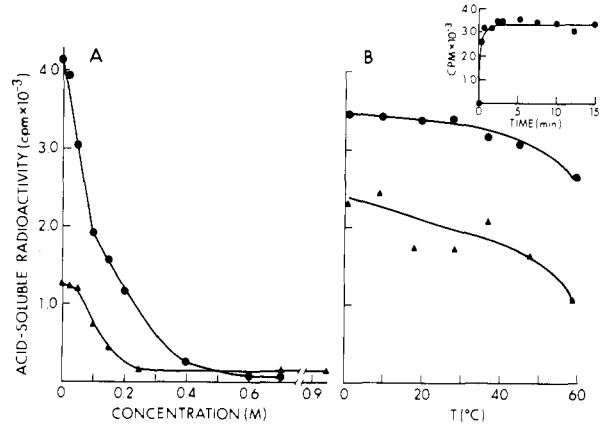

FIGURE 4: Effect of ionic strength and temperature on the degradation of DNA by bleomycin and Fe(II). (A) One series of reactions contained 6.6 μM adenovirus [^{3}H]DNA (3900 cpm), 60 μg/mL bleomycin, 1 × 10^{-4} M Fe(II), and the indicated concentrations of phosphate buffer, pH 7.0 (●). A second series contained the same amounts of bleomycin, DNA, and Fe(II) but were conducted in 0.05 M Tris-HCl and the indicated concentrations of NaCl (▲). Both series were conducted in a final volume of 50 μL. (B) Reaction mixtures contained in a final volume of 50 μL: 4.8 μM adenovirus [^{3}H]DNA (3600 cpm), 0.05 M phosphate, pH 7.0, 10 (▲) or 50 μg/mL (●) bleomycin, and 1 × 10^{-4} M Fe(II). Solutions of Fe(II) and the other components of the reaction mixture were equilibrated separately at the temperature indicated for 3 to 5 min prior to initiating the reactions. The *inset* demonstrates the kinetic properties of a reaction conducted at 4 °C as described for Figure 2, at a bleomycin concentration of 50 μg/mL.

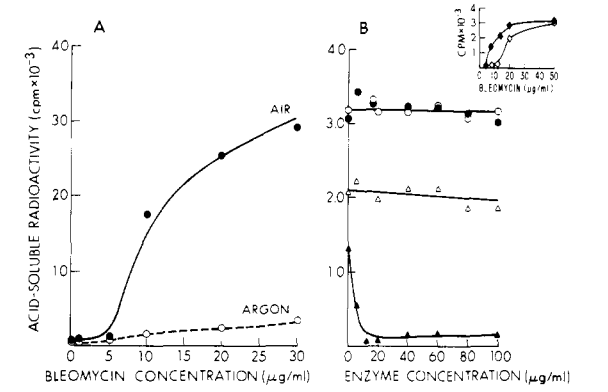

FIGURE 5: Participation of oxygen in the degradation of DNA by Fe(II) and bleomycin. (A) Reactions were conducted in septum-stoppered tubes with a sidearm in a final volume of 1.0 mL. The final concentration of reactants after addition of Fe(II) from the sidearm was 2.0 μM adenovirus [^{3}H]DNA (49 600 cpm), 0.05 M phosphate, pH 7.0, 1 × 10^{-4} M Fe(II), and the indicated concentrations of bleomycin. Reactions were conducted in the presence of argon (○) or air (●) at 22 °C for 10 min before addition of anaerobic EDTA with a gas-tight syringe to a final concentration of 0.05 M. Prior to addition of Fe(II) from a sidearm, reactions were equilibrated with air or argon for 20 min at 22 °C. (B) Reaction mixtures contained 4.4 μM adenovirus [^{3}H]DNA (3400 cpm), 0.05 M phosphate, pH 7.8, 1 × 10^{-4} M Fe(II), and 10 (▲) or 50 μg/mL (●) bleomycin and the indicated amounts of superoxide dismutase. Reactions conducted in the presence of catalase were run analogously, except that 12 (△) or 50 μg/mL (○) bleomycin was employed. The *inset* presents results obtained when the concentration of bleomycin was varied in the presence (◇) or absence (◆) of 16 μg/mL of superoxide dismutase.

kinetic experiments using adenovirus-2 [^{14}C]DNA and Geiger-Muller tube detection revealed qualitatively similar behavior to that described in Figure 3B (and in Figure 2). Despite the unusual characteristics of this effect, it is clear that the differences in extent of reaction between different buffers shown in Figure 3A cannot be attributed to simple kinetic differences in the course of such reactions.

Effect of Ionic Strength and Temperature. Figure 4A reveals that increasing the ionic strength significantly decreases the activity observed in the degradation of DNA by bleomycin and Fe(II), and the change occurs markedly between 0.05 and 0.3 M of buffer component. This result would be predicted from the finding that increased ionic strength decreases the binding of bleomycin to DNA (Chien et al., 1977). Also, the binding of Fe(II) to bleomycin could be reduced at high ionic strength.

The effect of temperature on the reaction is shown in Figure 4B. It can be observed that substantial activity occurs from 4 to 60 °C, with some decline in activity at higher temperatures. Inasmuch as bleomycin in aqueous solution is heat stable (Haidle et al., 1971), the decline in activity at higher temperatures cannot be attributed to simple inactivation of the drug under conditions used here. It may be related to a decreased ability of bleomycin to associate with DNA of increased single-stranded character. A decreased efficiency of breakage of single-stranded DNA by bleomycin has been documented (Onishi et al., 1975).

Effect of Oxygen. In a previous report from this laboratory (Sausville et al., 1976), the reaction occurring in the presence of bleomycin and Fe(II) with HeLa DNA was inhibited by the removal of oxygen. However, an ambiguity existed in that at higher concentrations of bleomycin significant activity of bleomycin and Fe(II) in the apparent absence of oxygen persisted. Appreciation of the kinetic properties of the reaction, however, as described in Figure 2, led to the use of gas-tight syringes for addition of EDTA to terminate these reactions. Also, the reactions were equilibrated with argon at the same temperature as incubation and utilized a three- to fourfold higher concentration of adenovirus DNA. On using these changes in procedure, the experiments of Figure 5A demonstrate that the removal of oxygen from this system effects virtually complete inhibition of the reaction which produces acid-soluble degradation products. It should be noted that in the aerobic reaction described in Figure 5A only about 60% of the DNA becomes acid soluble in a reaction that contains a tenfold molar excess of bleomycin to DNA and excess Fe(II) in relation to bleomycin. This apparent reduction in activity as compared to Figure 1B is attributed to the large volume of

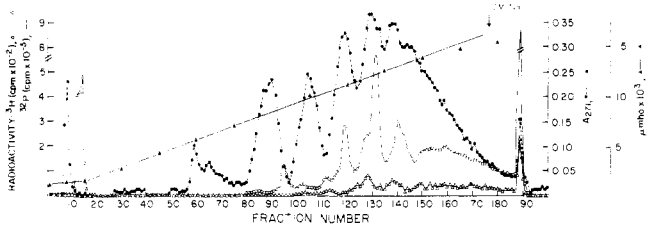

FIGURE 6: DEAE-cellulose chromatography of the limit product of Fe(II)-bleomycin degradation of DNA. The reaction mixture contained in a volume of 1.0 mL: 5 mM phosphate, pH 7.0, 22 μM adenovirus-2 [*thymine*-^{3}H,^{32}P]DNA, 333 μg/mL bleomycin, and 2 × 10^{-4} M Fe(II). The solution was saturated with O_2 prior to the start of reaction; incubation was for 1 h, 37 °C, with readdition of bleomycin and Fe(II) at 0.5 h (to a final concentration of 550 μg/mL and 4.2 × 10^{-4} M, respectively). At the end of reaction, the solution was made 0.01 M in EDTA, and to it was added 8.8 mg of calf thymus DNA which had been extensively digested with pancreatic DNase. After dilution to an appropriate buffer concentration (0.02 M) and addition of urea to 7 M, the sample was applied to a 1.8 × 27 cm column of DEAE-cellulose equilibrated with 0.02 M Tris-HCl, pH 7.6, 7 M urea. After washing with 60 mL of the latter buffer, nucleotides were eluted with a 0–0.3 M NaCl gradient (2 L), and fractions of 11 mL were collected at 1 mL/min. Nucleotides not eluted with the gradient were removed by 2 M NaCl in 0.02 M Tris-HCl, pH 7.6, 7 M urea. A_{271} (●), mμho (▲), [^{32}P] (○), and [^{3}H] (△) in aliquots of column fractions are shown.

the reaction in Figure 5A. The availability of oxygen to this reaction is diffusion limited in this circumstance.

Figure 5B shows that superoxide dismutase is capable of inhibiting the reaction only at a low concentration of bleomycin (10 μg/mL); at a higher concentration of bleomycin (50 μg/mL) the enzyme is without effect. Catalase does not inhibit the reaction under either of these conditions.

Products of the Degradation of DNA by Bleomycin and Fe(II)

Release of Free Bases. Reaction mixtures contained in a volume of 2 mL the following components: 2.3 × 10^{-4} M each of calf thymus DNA, bleomycin, and Fe(II) in 0.019 M phosphate buffer, pH 7.0. Fe(II) was added after the other components of the reaction mixture had been equilibrated with O_2 for 20 min at 22 °C. After addition of Fe(II) and continued incubation for 20 min at 22 °C, the release of free bases was detected by high-pressure liquid chromatography. This analysis (five assays) revealed that 13.7 ± 1.9% of thymine, 7.8 ± 0.8% cytosine, 4.2 ± 0.6% of adenine, and 2.8 ± 1.2% of guanine were released from DNA. Thus, pyrimidines are released more readily than purines and on the average one in every four bases in the DNA is released under these conditions.

Formation of Oligonucleotides. The products of a limit digest of adenovirus-2 [*thymine*-^{3}H,^{32}P]DNA with excess bleomycin and Fe(II) are presented in Figure 6. During this reaction 84% of the DNA present as ^{32}P became acid soluble. Fractionation was carried out on a DEAE-cellulose column in the presence of 7 M urea; elution of oligonucleotides occurs in order of increasing size (Tomlinson and Tener, 1963). The recovery of ^{32}P from this column was 95%. The ^{32}P-labeled oligonucleotides derived from the reaction of DNA with bleomycin and Fe(II) elute with a pattern quite distinct from that of the marker nucleotides derived from calf thymus DNA after treatment with pancreatic DNase (Figure 6). Fractions with appreciable ^{32}P are present which comigrate with marker oligonucleotides of chain length five, six, and seven (Table I). An unresolved fraction of ^{32}P is eluted which is greater than seven residues in length. A significant fraction of the applied label elutes with 2 M NaCl. This latter fraction has been sized by electrophoresis in urea-acrylamide gels and consists of

TABLE I: Size Distribution of Oligonucleotides Produced in a Limit Digest of Adenovirus-2 DNA by Bleomycin and Fe(II).[a]

col fract	size of oligonucl	fract of eluted ^{32}P
28–40	1	<0.001
60–70	2	<0.001
92–100	3–4	0.044
114–124	5	0.14
125–135	6	0.20
136–147	7	0.13
148–187	8–10	0.37
188–190	10–20	0.09

[a] The fraction of total radioactivity eluted from the DEAE-cellulose–urea column of Figure 6 migrating with marker nucleotides from a pancreatic DNase digest of a given average size class is tabulated. Assignment of chain length to marker nucleotides derived from pancreatic DNase treatment of calf thymus DNA is according to Tomlinson and Tener (1963).

oligonucleotides of size greater than 10 but less than 20 residues (data not shown). In a separate experiment in which the DNA was labeled to higher specific activity with [^{3}H]thymine, the material eluting in 2 M NaCl is not, after desalting, a substrate for further action of bleomycin and Fe(II). It is completely refractory to further release of thymine. Also, the ^{3}H species present in fractions 10–16 comigrates with thymine using the high-pressure liquid chromatography technique employed above (data not shown). From the distribution of the ^{32}P label given in Table I, we estimate that the median oligonucleotide produced after incubation of DNA with bleomycin and Fe(II) has a size of between seven and ten nucleotide residues. In an analogous reaction to that described in Figure 6, 98% of the ^{32}P label remained in a form that adsorbed to Norit after reaction of [^{32}P]DNA with bleomycin and Fe(II). When the reaction was conducted at higher ionic strength and equilibrated with air so that less than 50% of the DNA was rendered acid soluble, the nucleotides migrating in fraction 90–160 decreased; most of the applied radioactivity eluted with 2 M NaCl. We conclude that bleomycin, when acting on DNA with Fe(II), can produce discrete size classes of oligonucleotides without the generation of inorganic phosphate or mononucleotides.

The data in Table II examines the susceptibility of nucleotides from fractions 132–134 of Figure 6 to digestion by nucleases. The ^{32}P-labeled oligonucleotides of these fractions comigrated with pancreatic DNase-derived oligonucleotides of six residues in length $(pX)_6$. The terminal to internal phosphorus ratio (Table II) of these species would, however, be more consistent with structures of the type $(pX)_4$, $(pX)_6p$, or $(pX)_7p$. Indication of the unlikelihood of such structures is obtained from the experiments of Table II, which demonstrate that oligonucleotides from Fe(II)-bleomycin-treated DNA are refractory to complete digestion by spleen and venom exonucleases in the presence or absence of prior BALP treatment. Venom exonuclease causes degradation from a 3′-OH terminus of oligonucleotides, whereas spleen exonuclease requires a 5′-OH terminus. Control reactions utilizing a pancreatic DNase digest of adenovirus [^{32}P]DNA display the expected pattern of sensitivity to these nucleases. The experiments of Table III examine the oligonucleotides from fractions 139–142 of Figure 6. What is notable is that treatment of these species with venom exonuclease without prior BALP treatment causes an appreciable but incomplete fraction of the ^{32}P to become susceptible to BALP. Pancreatic DNase, which introduces 5′-P, 3′-OH breaks endonucleolytically, does not alter this

TABLE II: Characterization of Oligonucleotides Eluting in Fractions 132-134 of Figure 6.[a]

treatment	fract of ^{32}P not adsorbed to Norit: bleomycin-Fe(II) derived fragment	fract of ^{32}P not adsorbed to Norit: pancreatic DNase digest
(1) none	0.02	0.02
(2) BALP	0.27	0.21
(3) heat, BALP	0.31	0.22
(4) venom exonuclease, heat, BALP	0.38	0.98
(5) spleen exonuclease, heat, BALP	0.31	0.22
(6) BALP, venom exonuclease, BALP	0.48	1.00
(7) BALP, spleen exonuclease, BALP	0.73	0.96

[a] Column fractions were desalted as described under Materials and Methods; a pancreatic DNase digest of adenovirus-2 [^{32}P]DNA was used directly. The nucleotides were treated sequentially with enzymes in the following manner. In all cases, appropriate control experiments demonstrated that the values shown represent complete reaction. Reaction 2 contained Fe(II)-bleomycin-derived fragments (512 cpm, 6.4×10^{-4} M in nucleotide equivalents) or the oligonucleotides from a pancreatic DNase digest of adenovirus-2 DNA (1733 cpm, 8.6×10^{-6} M), 0.02 M Tris-HCl, pH 8.0, and BALP, 20 units/mL in a volume of 50 μL. Incubation was for 1 h, 37 °C, and Norit nonadsorbable radioactivity determined. Reaction 6 was conducted by adjusting reaction 2 to the following concentrations: 0.04 M Tris-HCl, pH 8.8, 0.12 M $MgCl_2$, and 14 units/mL venom exonuclease in a final volume of 100 μL. Reaction was allowed to proceed for 2 h, 37 °C, whereupon Tris-HCl was added to a final concentration of 0.1 M, pH 8.0, and BALP readded to a final total concentration of 22 units/mL in a final volume of 120 μL. Incubation continued for 1 h, 37 °C, and Norit nonadsorbable radioactivity determined. Reaction 7 was conducted by adjusting reaction 2 to the following concentrations: 0.1 M acetate, pH 6.0, and 3.2 units/mL spleen exonuclease in a volume of 100 μL. Digestion proceeded for 2 h, 37 °C, whereupon the solutions were made 0.53 M in Tris-HCl, pH 8.0, and BALP was readded to a final concentration of 36 units/mL in a volume of 200 μL. Incubation was continued for 1 h, 37 °C, and Norit nonadsorbable radioactivity determined. Reactions 4 and 5 were conducted like reactions 6 and 7, except that addition of BALP prior to addition of nucleases was omitted, and the reaction mixtures were heated at 95 °C for 5 min after treatment with nucleases prior to addition of BALP to a final concentration of 14 units/mL (after venom exonuclease) or 30 units/mL (after spleen exonuclease). Reaction 3 was conducted as reaction 4, except that treatment with venom exonuclease was omitted.

TABLE III: Characterization of Oligonucleotides Eluting in Fractions 139-142 of Figure 6.[a]

treatment	fraction of ^{32}P not adsorbed to Norit: bleomycin-Fe(II) derived fragment	fraction of ^{32}P not adsorbed to Norit: adenovirus DNA
Series A		
(1) none	0.01	0.01
(2) BALP	0.25	0.01
(3) pancreatic DNase, BALP	0.25	0.20
(4) venom exonuclease, BALP	0.60	0.16
(5) pancreatic DNase, venom exonuclease, BALP	0.59	0.98
Series B		
(6) pancreatic DNase, BALP, venom exonuclease (1 h), BALP	0.66	0.97
(7) as in 6, but venom exonuclease (1.5 h)	0.68	1.02
(8) as in 6, but venom exonuclease (2 h)	0.72	0.95

[a] Column fractions were pooled and desalted as under Materials and Methods; adenovirus [^{32}P]DNA was used directly. The nucleotides were treated sequentially with enzymes in the following manner. In Series A, reaction 5 contained Fe(II)-bleomycin-treated fragments (496 cpm, 7.5×10^{-4} M nucleotides) or adenovirus [^{32}P]DNA (19 956 cpm, 2.2×10^{-5} M nucleotides), 0.005 M Tris-HCl, pH 7.6, 0.005 M $MgCl_2$, and 20 μg/mL pancreatic DNase in a final volume of 50 μL. After 1 h at 37 °C, the reactions were adjusted to a final concentration of 0.02 M Tris-HCl, pH 8.8, 0.09 M $MgCl_2$, and 14 units/mL venom exonuclease in a final volume of 100 μL; incubation was continued for 1 h, 37 °C. Tris-HCl was then added to a final concentration of 0.12 M, pH 8.0, and BALP to a final concentration of 14 units/mL. Incubation was for 1 h, 37 °C, and Norit nonadsorbable radioactivity was determined. Reactions 2-4 were conducted analogously, except the appropriate component was not added. In series B, after treatment with pancreatic DNase as in series A, reactions were made 0.015 M Tris-HCl, pH 8.0, and 23 units/mL BALP. Incubation was for 45 min, 37 °C. Reactions were then made 0.033 M in Tris-HCl, pH 8.8, 0.105 M $MgCl_2$, and 12 units/mL venom exonuclease. Incubation was at 37 °C for the times indicated. The reactions conducted for 1.5 and 2 h received an additional aliquot of venom exonuclease after 1 h. The reactions were made 0.12 M in Tris-HCl, pH 8.0, and BALP was readded to final total concentration of 22 units/mL. After incubation at 37 °C for 1 h, the amount of Norit nonadsorbable radioactivity was determined.

result. This behavior is to be contrasted with the response of the nucleotides of Table II to venom exonuclease without prior BALP treatment. The data of series B, Table III, indicate that treatment with BALP before venom exonuclease increases the sensitivity of this oligonucleotide fraction to the nuclease by less than 15%. We conclude from the experiments of Tables II and III that the oligonucleotide fractions derived from Fe(II)-bleomycin digests of DNA display several features, which suggest that they may be quite heterogeneous and are not simple structures such as those deriving from uncomplicated nucleolytic digestion of DNA.

A reaction mixture virtually identical to that described in Figure 6 was subjected to exhaustive dialysis and then treated with pancreatic DNase and venom exonuclease without prior passage through a urea-DEAE-cellulose column. Thin-layer chromatography revealed several oligonucleotides in addition to the four 5′-deoxynucleotides, whereas non-Fe(II)-bleomycin-treated DNA was converted completely to mononucleotides (data not shown). Thus, the results of Tables II and III are not artifacts resulting from the passage of these oligonucleotides through the column or from the desalting procedures.

Formation of Aldehydes. A characteristic product of the reaction of bleomycin with DNA in the presence of organic reducing agents is a species which resembles malondialdehyde. This substance had been detected by Kuo and Haidle (1974) after long periods of reaction of calf thymus DNA with bleomycin in the presence of 2-mercaptoethanol. Figure 7A demonstrates that the reaction of bleomycin and Fe(II) with DNA generates a species which reacts with 2-thiobarbituric acid and that the occurrence of this species is dependent on the presence of DNA, bleomycin, and Fe(II). Fe(III) cannot substitute for Fe(II). The optical spectral characteristics of the chromophore derived from 2-thiobarbituric acid and this species in the region of 600-500 nm are identical to the chromophore obtained when 2-thiobarbituric acid reacts with authentic malondialdehyde (not shown). We do not, however, feel that this necessarily implies that malondialdehyde is generated in this reaction. What is notable in the data of Figure 7A is that

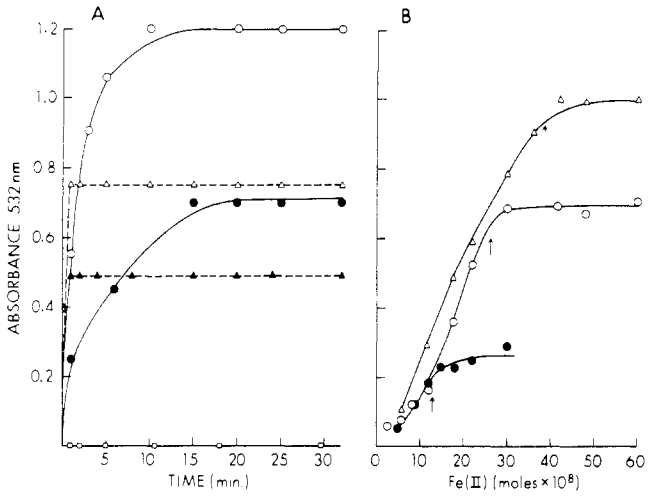

FIGURE 7: Generation of a malondialdehyde-like product after reaction of bleomycin and Fe(II) with DNA. (A) The complete reaction mixture contained in a final volume of 2.2 mL in septum-stoppered test tubes: 0.019 M phosphate buffer, pH 7.0, and 0.23 mM each of calf thymus DNA, bleomycin, and Fe(II). Prior to addition of Fe(II) the reactions were equilibrated at 21 °C with the atmospheres indicated below and then brought to the indicated temperature. Fe(II) was in all cases equilibrated with air at the temperature of the reaction and added in a small volume to initiate the reaction. At the times indicated, a 0.2-mL aliquot of the reaction mixture was assayed with 2-thiobarbituric acid as described under Materials and Methods. Conditions employed were as follows: complete reaction, 37 °C, in air (▲) or O_2 (△); complete reaction at 37 °C in air where Fe(III) is substituted for Fe(II) or where Fe(II), bleomycin, or DNA is omitted (□); complete reaction, 4 °C, in air (●) or O_2 (○). (B) Reactions contained in a volume of 1.3 mL: 0.019 M phosphate, pH 7.0, 2.9×10^{-3} M calf thymus DNA, Fe(II) in the indicated amount, and 155 (0.13 μmol, ●), 310 (0.26 μmol, ○), or 465 μg/mL (0.39 μmol, △) bleomycin. Reactions were initiated by addition of Fe(II) at 37 °C. Iron was added rapidly in a volume of 0.13 mL, and the tubes were shaken for 15–30 s to effect mixing. Incubation was continued for 15 min. An aliquot was removed from each reaction mixture for the determination of malondialdehyde-like material. The arrows indicate the amount of Fe which is approximately equimolar to the bleomycin present in reaction mixtures.

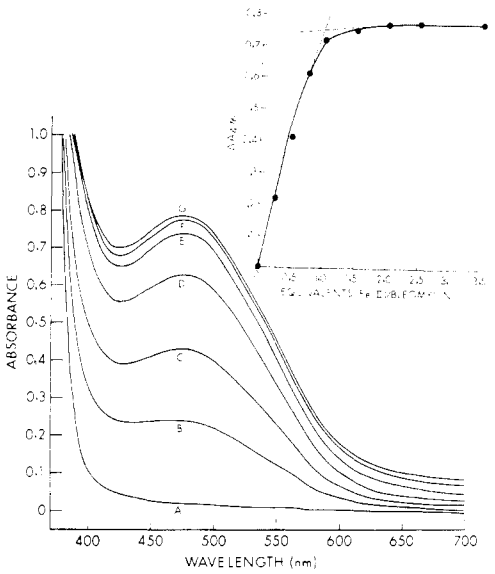

FIGURE 8: Anaerobic titration of bleomycin with Fe(II). In the main compartment of a thunberg cuvette, a solution (3 mL) consisted of: 2 mM bleomycin, 0.05 M Hepes, pH 7, plus 5–8 mg of dithionite in an argon atmosphere as described under Materials and Methods. To this solution were added increments of anaerobically prepared Fe(II) to the following final concentrations (M): A, 0; B, 5×10^{-4}; C, 1×10^{-3}; D, 1.5×10^{-3}; E, 2×10^{-3}; F, 3×10^{-3}; G, 4×10^{-3}, 5×10^{-3} and 7×10^{-3}. The *inset* plots the observed ΔA_{476} as a function of the ratio of Fe(II) to bleomycin.

the generation of this species at 37 °C occurs with a time course that closely resembles the kinetics of DNA degradation to acid-soluble products (Figure 2). A greater amount of this species is formed when the reaction is conducted in the presence of elevated oxygen tension. Although the temperature dependence of the kinetics of this species' production is somewhat different from that described in Figure 4, it is apparent that the reaction of Fe(II) and bleomycin with DNA as assayed by this method still proceeds with remarkable rapidity even at 4 °C. The increased amount of this aldehyde species produced at 4 °C in comparison to 37 °C may be related to the increased solubility of O_2 in solutions of lower temperature.

The generation of the malondialdehyde-like substance allows an independent approach to the stoichiometry of Fe(II) with respect to bleomycin in the degradation of DNA. Reactions were conducted in the presence of an excess of DNA in relation to bleomycin, with varying amounts of added Fe(II). Figure 7B demonstrates that at three concentrations of bleomycin the production of a malondialdehyde-like substance reaches a plateau at an amount of Fe(II) which is approximately equivalent to the moles of bleomycin present in the reaction.

Properties of Fe(II)–Bleomycin

In view of the extensive analysis of the properties and products of the degradation of DNA by bleomycin and Fe(II), it is of interest to examine whether these results can be correlated with the properties of the complex between Fe(II) and bleomycin formed in the absence of DNA, whose lability to oxygen has been described previously (Sausville et al., 1976).

Stoichiometry. Titration of bleomycin with Fe(II) under scrupulously anaerobic conditions demonstrates that a 1:1 complex is formed between the antibiotic and Fe(II), with λ_{max} 476 nm and ϵ_M (apparent) = 3.8×10^2 (Figure 8). This result assumes significance in light of the results presented in Figure 7B, where the generation of a malondialdehyde-like product was observed to occur with the same apparent stoichiometry. It is therefore most probable that the spectrophotometrically observed Fe(II)–bleomycin complex is directly relevant to the degradation of DNA.

Effect of pH. Figure 9 demonstrates that the Fe(II)–bleomycin complex is sensitive to decreasing pH between 6.2 and 4.3 with a pK of 5.2 in acetate buffer. This result should be compared with Figure 3A, where it can be seen that activity of Fe(II)–bleomycin in causing the degradation of DNA to acid-soluble products also disappears between pH 6.2 and 4.3 in acetate buffer. Thus, the titration of Fe(II)–bleomycin correlates directly with the loss of activity in the degradation of DNA. It is of interest to point out that the chemical shift of protons from the β-hydroxyhistidine residue of metal-free bleomycin has an apparent pK of 5.4 (in D_2O) (Chen et al., 1977). Whether this is also the residue in Fe(II)–bleomycin with pK = 5.2 must await further study.

Discussion

The results presented in this paper demonstrate that Fe(II) and bleomycin are capable of causing the extremely efficient degradation of DNA. Under appropriate conditions the reaction can proceed virtually to completion in less than 1 min. The products of degradation include all four DNA bases, discrete oligonucleotides, and a malondialdehyde-like product. The reaction is dependent on the presence of molecular oxygen. Marked buffer effects are observed with our acid-solubility assay, although an absolute dependence on pH is not apparent

between pH 4.5 and 10.5. Between 2 and 50 °C, temperature is not an important variable.

The principal rationale for conducting these experiments was to demonstrate that the reaction of bleomycin with Fe(II) could account for product distribution which has been described using organic reducing agents with bleomycin. This was deemed of importance in view of the considerable evidence (Sausville et al., 1978) that it is through contaminating Fe(II) that these organic reducing agents act with bleomycin to cleave DNA. Haidle et al. (1972) demonstrated that bleomycin and 2-mercaptoethanol could cause the release of all four bases from DNA, although the relative amount of release was not quantitated. These workers also showed that mononucleotides and inorganic phosphate were not released in the reaction. Our data with Fe(II) indicate that pyrimidine bases are released preferentially in relation to purines and neither inorganic phosphate nor mono- or dinucleotides are produced. Kuo et al. (1973) described a bleomycin-refractory species of about 10–13 nucleotides in length that was generated in the limit of bleomycin action with 2-mercaptoethanol. Our experiments demonstrate that the limit product of bleomycin and Fe(II) is on the average 7–10 nucleotides in length, although discrete species are formed with a range of sizes from 3–4 to 10–20 residues in length. The data of Kuo et al. (1973) may be interpreted as indicating a refractoriness of their limit product to the action of common nucleases; our experiments demonstrate conclusively that oligonucleotides derived from the degradation of DNA by bleomycin and Fe(II) are highly atypical with respect to their sensitivity to these nucleases.

The nature of this refractoriness to nuclease digestion could extend from a number of sources. The ends of these species could be blocked by carbons originating from deoxyribose moieties at the site of base release. Alternatively, if aldehyde species are generated at the ends of the oligonucleotides then these residues could condense with any of a number of amine-containing species (including bleomycin) to give blocked oligonucleotides of a different type. Another possibility extends from the probable existence of apurinic or apyrimidinic sites in these oligonucleotides. The relative susceptibility of such structures to nucleolytic attack with nucleases has not been rigorously established. Finally, Fe(III) deriving from oxidation of Fe(II) may interfere with the action of nucleases, especially if complexed with oligonucleotides. Any of these possible situations would be expected to produce oligonucleotides whose refractoriness to nucleases would not be relieved by prior treatment with BALP.

The aldehyde product which resembles malondialdehyde and which is formed after extensive reaction of DNA with bleomycin in the presence of 2-mercaptoethanol (Kuo and Haidle, 1974) is also apparently formed by bleomycin and Fe(II). Despite the similarity of this species to malondialdehyde, as revealed by the chromophore produced with 2-thiobarbituric acid, positive identification of this species must await further study. This problem extends from the fact that other aldehyde species, including α,β unsaturated aldehydes and acetaldehyde in the presence of sucrose (Schmidt, 1959; Baumgartner et al., 1975), are known to give with 2-thiobarbituric acid a closely analogous chromophore to that obtained with malondialdehyde. Despite this difficulty, a result of particular significance which has emerged from these studies is that the stoichiometry of Fe(II) and bleomycin in producing this aldehyde is 1:1. This stoichiometry is also observed when the formation of Fe(II)–bleomycin is followed spectrophotometrically in the absence of DNA. This is strong evidence that the degradation of DNA observed here is specifically related to the formation of this complex. The pH dependence of the

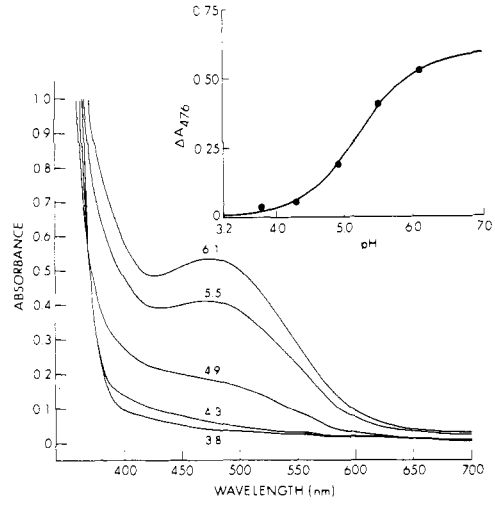

FIGURE 9: pH dependence of Fe(II)–bleomycin. As in Figure 8, except that bleomycin solutions were in 0.05 M acetate at the indicated pH, and Fe(II) was added from the sidearm to a concentration of 2×10^{-3} M after deoxygenation and addition of dithionite as described under Materials and Methods. The *inset* plots ΔA_{476} as a function of pH. This curve was fit to the data as described under Materials and Methods.

reaction as compared to the pK for Fe(II)–bleomycin is further evidence for this viewpoint.

Our data indicate that the pattern of base release from calf thymus DNA is not what would be expected from the random release of bases. Pyrimidines are released to a greater extent than purines, and thymine is released more efficiently than cytosine. The fact that this is a very similar pattern of base release to that obtained by Takeshita et al. (1977) using HeLa DNA reacted with bleomycin in the presence of 2-mercaptoethanol and Mg(II)–ATP underscores the probability that Fe(II) is the active species in systems containing organic reducing agents which act with bleomycin to degrade DNA. A distinct pattern of base release indicates an element of specificity in the reaction of DNA with Fe(II)–bleomycin. This specificity could derive from at least two sources. The oxidation of Fe(II)–bleomycin bound to DNA could produce species to which pyrimidine residues have a greater intrinsic sensitivity than purine residues. Also, the affinity of bleomycin for particular sequences in DNA could determine what base residues are exposed to the products of this oxidative event. It is possible, therefore, that the binding specificity of the drug or drug–metal complex may be quite distinct from the breaking specificity of intermediates produced in the oxidation of the drug–metal complex. The ultimate pattern of base release observed would be a function of both aspects of the interaction of Fe(II)–bleomycin with DNA.

Certain features of the degradation of DNA by Fe(II)–bleomycin require comment. Although the highly efficient degradation of DNA by Fe(II)–bleomycin requires oxygen, just as the reaction described in the absence of added Fe(II) (Onishi et al., 1975), our reaction is not inhibited by superoxide dismutase or catalase at high concentrations of drug. This is in accord with the finding of Ishida and Takahashi (1975), who examined the degradation of DNA by bleomycin in the presence of 2-mercaptoethanol. The finding that these scavengers of $\cdot O_2^-$ and H_2O_2 did not inhibit this oxygen-dependent reaction is not surprising in view of the proposal that radical species relevant to the degradation of DNA and derived from oxygen may be formed at the site of Fe(II)–bleomycin binding to DNA (Sausville et al., 1978). Diffusion of these species would not be expected to occur as a necessary part of their potential role in mediating damage to DNA. The reason for

inhibition of the reaction at low concentrations of bleomycin by superoxide dismutase is not clear. Superoxide dismutase may nonenzymatically interfere with the formation of Fe(II)-bleomycin complex or with the binding of bleomycin to DNA. This tendency would be overcome by higher concentrations of bleomycin. Alternatively, at low concentrations of bleomycin, diffusible $\cdot O_2^-$ may be an important participant in the reaction.

The difference observed between the extent of reaction in different buffers is quite striking (Figure 3). While it is possible that different buffers may alter the level of free Fe(II), the kinetics of the degradation of DNA by bleomycin and Fe(II) in Tris or acetate buffers are most unusual (Figure 3B) and are not observed when the degradation reaction is carried out in the presence of Tris-HCl, 2-mercaptoethanol, and Mg-ATP, without addition of Fe(II) (Takeshita et al., 1976). This phenomenon could represent an alteration of the size of the oligonucleotide initially produced in the reaction. In this connection, Ogilvie and Whitaker (1976) have demonstrated that Tris buffer greatly alters the kinetics of enzymatic reactions of aldehydes, probably by condensation with these substrates. Such reactions occurring between amino groups and aldehydes present in our reaction mixtures could lead to a condensed polymer that does not reflect the true size of oligonucleotides produced immediately after reaction with Fe(II)-bleomycin. An alternative and perhaps more likely explanation of this effect arises from the experiments of Spiro et al. (1966), who demonstrated that under certain circumstances the hydrolysis of Fe(III) can give rise to a polymer of up to 1.4×10^5 daltons. If this process were to occur in a differing extent in Tris or acetate buffers as opposed to phosphate buffer, the involvement of product oligonucleotides in this polymerization might alter their solubility in acid as determined by our assay.

The dependence of the reaction of DNA with Fe(II) and bleomycin on pH differs from the pH dependence observed in the absence of reducing agents (Umezawa et al., 1973) and in the presence of Mg(II)-ATP (Takeshita et al., 1977). In both of these studies substantial activity below pH 7 was not observed, in contrast to our results. It should be noted, however, that the former workers used a citrate buffer in this range of pH. Citrate is an effective chelator of Fe and may inhibit the activity of bleomycin. In the latter studies ATP was present, which also forms a complex with Fe (Goucher and Taylor, 1964) and may alter the distribution of Fe to bleomycin in the course of the reaction.

In conclusion, it is apparent that with respect to products formed and several characteristics of the reaction, Fe(II) and bleomycin act to degrade DNA in a way that is easily reconciled with previous studies of the degradation of DNA by bleomycin in the presence of high concentrations of organic reducing agents. We feel that these data, in conjunction with evidence already described (Sausville et al., 1976, 1978), indicate quite strongly that Fe(II) is a necessary cofactor of bleomycin in degrading DNA, as this reaction is usually conducted in vitro. Recognition that contaminating Fe(II) probably acts with bleomycin to degrade DNA even in reactions to which this metal ion is not added will be of assistance in designing and interpreting future studies with this antibiotic.

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