Cleavage of DNA with Methidiumpropyl-EDTA–Iron(II): Reaction Conditions and Product Analyses†

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ABSTRACT: The synthesis of methidiumpropyl-EDTA (MPE) is described. The binding affinities of MPE, MPE·Ni(II), and MPE·Mg(II) to calf thymus DNA are 2.4×10^4 M^{-1}, 1.5×10^5 M^{-1}, and 1.2×10^5 M^{-1}, respectively, in 50 mM NaCl, pH 7.4. The binding site size is two base pairs. MPE·Mg(II) unwinds PM2 DNA 11 ± 3° per bound molecule. MPE·Fe(II) in the presence of O_2 efficiently cleaves DNA and with low sequence specificity. Reducing agents significantly enhance the efficiency of the cleavage reaction in the order sodium ascorbate > dithiothreitol > NADPH. At concentrations of 0.1–0.01 μM in MPE·Fe(II) and 10 μM in DNA base pairs, optimum ascorbate and dithiothreitol concentrations for DNA cleavage are 1–5 mM. Efficient cleavage of DNA (10 μM in base pairs) with MPE·Fe(II) (0.1–0.01 μM) occurs over a pH range of 7–10 with the optimum at 7.4 (Tris-HCl buffer). The optimum cleavage time is 3.5 h (22 °C). DNA cleavage is efficient in a Na^+ ion concentration range of 5 mM to 1 M, with the optimum at 5 mM NaCl. The number of single-strand scissions on supercoiled DNA per MPE·Fe(II) under optimum conditions is 1.4. Metals such as Co(II), Mg(II), Ni(II), and Zn(II) inhibit strand scission by MPE. The released products from DNA cleavage by MPE·Fe(II) are the four nucleotide bases. The DNA termini at the cleavage site are 5′-phosphate and roughly equal proportions of 3′-phosphate and 3′-(phosphoglycolic acid). The products are consistent with the oxidative degradation of the deoxyribose ring of the DNA backbone, most likely by hydroxy radical.

Judicious attachment of EDTA·Fe(II)[1] to a DNA binding molecule affords a DNA cleaving molecule (Hertzberg & Dervan, 1982). Methidiumpropyl-EDTA (MPE) **3**, which contains the metal chelator ethylenediaminetetraacetic acid (EDTA) tethered to the DNA intercalator methidium, cleaves DNA efficiently in a reaction dependent on ferrous ion and oxygen. Addition of reducing agents such as dithiothreitol (DTT) increases the efficiency of DNA cleavage (Hertzberg & Dervan, 1982). MPE·Fe(II) cleaves DNA in a relatively non-sequence-specific manner and with significantly lower sequence specificity than the enzyme DNase I (Van Dyke & Dervan, 1982, 1983ab). MPE·Fe(II) is a useful DNA cleaving agent for determining the locations, size, and relative importance of the binding sites of small molecules on native DNA (Van Dyke et al., 1982; Van Dyke & Dervan, 1982, 1983ab). MPE·Fe(II) footprinting has been utilized to determine the binding locations and site sizes of actinomycin, distamycin, chromomycin, mithramycin, olivomycin, and echinomycin on several restriction fragments from pBR322 plasmid DNA (Van Dyke et al., 1982; Van Dyke & Dervan, 1982, 1983a,b, 1984). In addition, MPE·Fe(II) is a useful DNA cleaving agent in the study of chromatin structure (Cartwright et al., 1983).

We report here the synthesis, purification, and characterization of MPE (**3**), the binding affinities of MPE, MPE·Mg(II), and MPE·Ni(II) to calf thymus DNA, the unwinding of superhelical DNA by MPE·Mg(II), reaction conditions for DNA cleavage, factors affecting the cleavage efficiency of DNA by MPE·Fe(II), and analyses of the DNA cleavage products.

Materials and Methods

Materials. Blenoxane, a clinical bleomycin sulfate, was generously supplied by Bristol Laboratories. Thiobarbituric acid (TBA) and nucleotide bases were from Sigma. Calf intestinal alkaline phosphatase was obtained from Boehringer Mannheim. DNase I was from Worthington, topoisomerase I was from Bethesda Research Labs, and all other enzymes were from New England Biolabs. Amersham supplied [^{32}P]dATP and [^{3}H]thymidine.

Synthesis of Methidiumpropylamine **2**. *p*-Carboxymethidium chloride **1** (Dervan & Becker, 1978) (1.0 g, 2.6 mmol) and *N*-ethylmorpholine (0.3 mL) were combined in 40 mL of dry Me_2SO under an argon atmosphere. Carbonyldiimidazole (470 mg, 2.9 mmol) in 6 mL of Me_2SO was added at room temperature, and the solution was allowed to stir for 1 h. The contents of the flask were transferred via syringe to a dropping funnel and added dropwise to a solution of 1,3-diaminopropane (2.2 mL, 26 mmol) in 2 mL of dry Me_2SO under argon. Stirring was maintained for 24 h, followed by concentration in vacuo to yield a purple solid. The solid was flash chromatographed on silica gel 60 with acidic methanol [0.1% (v/v) acetyl chloride in dry methanol]. A dark orange band was collected, concentrated in vacuo, and dried for several days in vacuo at 50 °C to yield 998 mg (89%) of the maroon solid **2**: NMR (D_2O) δ 7.19–8.07 (m, 10 H, aromatic), 4.19 (s, 3 H, N^+–Me), 3.63 (t, J = 7 Hz, 2 H, $-CH_2-$), 3.20 (t, J = 7 Hz, 2 H, $-CH_2-$), 2.13 (m, 2 H, $-CH_2-$).

Synthesis of Methidiumpropyl-EDTA Triethyl Ester **5**. Triethyl ethylenediaminetetraacetate (**4**) (119 mg, 0.32 mmol), prepared by the method of Hay & Nolan (1975), was combined with carbonyldiimidazole (57 mg, 0.35 mmol) in 3 mL of dry DMF and allowed to stir at room temperature for 30 min. Methidiumpropylamine **2** (138 mg, 0.32 mmol) was added, and the solution was allowed to stir at room temperature for 24 h. The mixture was concentrated in vacuo affording a red solid that was flash chromatographed on silica

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[1] Abbreviations: MPE, methidiumpropyl-EDTA; BLM, bleomycin; TBA, 2-thiobarbituric acid; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; Me_2SO, dimethyl sulfoxide; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; NADH, nicotinamide adenine dinucleotide, reduced form; bp, base pair.

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gel 60 with acidic methanol [0.2% (v/v) acetyl chloride in dry methanol] to yield 124 mg (49%) of the triester **5**: NMR (CD_3OD) δ 7.3–8.7 (m, 9 H, aromatic), 6.48 (d, J = 3 Hz, 1 H, H-7), 4.12 (m, 9 H, N^+–CH_3 and –$COOCH_2CH_3$), 3.2–3.7 (m, 12 H, –N–CH_2–CO– and CO–NH–CH_2–), 2.76 (s, 4 H, –N–CH_2–), 1.86 (m, 2 H, –CH_2–), 1.25 (t, J = 7 Hz, 9 H, –$COOCH_2CH_3$).

Synthesis of Methidiumpropyl-EDTA (MPE) **3**. *Method A*. EDTA (4.5 g, 15.4 mmol, free acid) was dissolved in 600 mL of dry DMF at 120 °c under an argon atmosphere. Molecular sieves were added, and methidiumpropylamine **2** (435 mg, 1 mmol) in 150 mL of DMF was added dropwise to the hot solution. The solution was allowed to stir for 1 h at 120 °C, cooled to room temperature, and filtered. The filtrate was concentrated in vacuo affording a red solid. This was dissolved in warm water and passed over Amberlite IRA45 to remove excess EDTA. The orange aqueous solution was concentrated in vacuo to yield a purple solid, which was flash chromatographed on silica gel 60 with basic methanol (2% aqueous NH_3 in methanol). A dark orange band was collected and concentrated in vacuo. The product was rendered metal free by dissolving it in 100 mL of 5% aqueous Na_2EDTA. This solution was acidified to pH 2 with 1 M HCl, neutralized with aqueous NH_3, and passed over a column of Amberlite XAD-2 polystyrene resin (Rohm and Haas) to effect adsorption of the product. This column was washed with 200 mL of chelex-treated metal-free 5% aqueous NaCl and 200 mL of double-distilled H_2O. Subsequent elution with 50% aqueous methanol yielded a red solution that was concentrated in vacuo to yield 475 mg (67%) of the maroon solid, MPE **3**: NMR (CD_3OD) δ 7.33–8.62 (m, 9 H, aromatic), 6.54 (s, 1 H, H-7), 4.12 (s, 3 H, N^+–Me), 3.90 (s, 4 H, N–CH_2–COOH), 3.05–3.55 (m, 12 H, N–CH_2 and CONH–CH_2–), 1.92 (m, 2 H, –CH_2–); IR (KBr) 3400, 3200 (sh), 2900, 1630, 1580, 1490, 1410, 1315, 1260, 1110, 820 cm^{-1}; UV (H_2O) 286 nm (ϵ 54725 M^{-1} cm^{-1}), 488 (ϵ 5994 M^{-1} cm^{-1}). MPE was isolated as the monopotassium salt tetrahydrate: mass spectrum, m/z 712 (monopotassium salt, M^+). Anal. Calcd for $C_{34}H_{47}N_7O_{12}K$: C, 52.03; H, 6.04; N, 12.49. Found: C, 52.24; H, 5.78; N, 12.46.

Method B. Triester **5** (124 mg, 0.16 mmol) was dissolved in 10 mL of ethanol, and 25 mL of 0.5 M lithium hydroxide was added. The reaction was allowed to stir at room temperature for 2 h, acidified to pH 4 with 1 M HCl, and concentrated in vacuo to a red solid. The product was flash chromatographed on silica gel and further purified as described under Method A to yield 101 mg (79%) of **3**. MPE prepared in this way was identical with that produced by method A by NMR, IR, TLC, and HPLC analyses.

Synthesis of Propane-EDTA **6**. Triester **4** (200 mg, 0.53 mmol) was combined with carbonyldiimidazole (95 mg, 0.58 mmol) in 5 mL of dry DMF and stirred at room temperature for 30 min. Propylamine (0.052 mL, 0.64 mmol) was added, and the solution was allowed to stir at room temperature for 24 h. The mixture was concentrated in vacuo to a yellow oil and taken up in 5 mL of ethanol. Lithium hydroxide (5 mL of 0.5 M) was added, and the reaction was allowed to stir at room temperature for 1 h, acidified to pH 7 with 1 M HCl, and concentrated in vacuo to a yellow oil. The product was flash chromatographed with basic methanol (20% aqueous NH_3 in methanol) to yield 101 mg (56%) of **6**: NMR (CD_3OD) δ 3.28 (m, 2 H, –CONH–CH_2–), 3.18 (s, 2 H, –N–CH_2–CONH–), 3.08 (s, 6 H, –N–CH_2–COOH), 2.57 (s, 4 H, –N–CH_2–), 1.53 (m, 2 H, –CH_2–), 0.92 (t, J = 7 Hz, 3 H, –CH_3).

DNA Substrates. Calf thymus DNA, purchased from Sigma, was sonicated, phenol extracted, and extensively dialyzed. PM2 plasmid DNA was from Boehringer Mannheim. pBR322 plasmid DNA was grown in *Escherichia coli* strain HB101 and isolated in supercoiled form by procedures similar to those of Tanaka & Weisblum (1974). Bacteriophage λ [^{3}H]DNA labeled at the 5-methyl group of thymine was purchased from Bethesda Research Labs. [^{3}H]DNA labeled at the 5′-position of thymidine was extracted from purified bacteriophage λ, grown in a thy$^-$ host, *E. coli* strain RS15, kindly provided by Richard Burger (Burger et al., 1980). This heat-inducible, lysis-defective bacteriophage λ lysogen was grown, and the phage was isolated as previously described (Maniatis et al., 1982). 5′-Labeled thymidine and 85 μg/mL uridine were added after induction. The DNase I treatment and cesium chloride step gradient were omitted. DNA was extracted from phage by formamide dialysis (Thomas & Davis, 1975) and extensively dialyzed with 10 mM Tris, pH 7.4/50 mM NaCl. The specific activity was 9.5 mCi/mmol of base pair.

^{32}P end-labeled DNA fragments of defined sequence were obtained from the bacterial plasmid pBR322 (Sutcliffe, 1979). A 279 base pair 3′-end-labeled DNA fragment was prepared by cleavage of the plasmid with *Bam*HI and enzymatic extension of the 3′-end with the Klenow fragment of DNA polymerase I and [α-^{32}P]dATP (3000 Ci/mmol) (Sanger & Coulson, 1975). After a second cleavage with *Sal*I, the fragment was isolated by gel electrophoresis on a 5% polyacrylamide, 1:30-cross-linked, 2 mm thick gel (Maxam & Gilbert, 1980). A 378 base pair 5′-end-labeled DNA fragment was prepared by cleavage of the plasmid with *Bam*HI followed by removal of the 5′-phosphoryl groups with calf intestinal alkaline phosphatase. The 5′-ends were labeled with [γ-^{32}P]ATP (5000 Ci/mmol) and T4 polynucleotide kinase (Maxam & Gilbert, 1980). After a second digestion with *Eco*RI, the DNA fragment was isolated from a 5% polyacrylamide gel.

DNA Binding Affinities. The absorbance titrations were performed with a Cary 219 spectrophotometer in 10 cm long cells (25 mL) at 23 ± 1 °C. The buffer was 10 mM Tris-HCl/50 mM NaCl, pH 7.4. Increasing amounts of MPE were added to a known quantity of sonicated calf thymus DNA, and the absorbance at 488 nm was recorded until equilibrium was attained (10 min). Absorbance measurements were reproducible to ±0.0004 AU. The extinction coefficients of free and bound MPE at 488 nm are 5994 M^{-1} cm^{-1} and 2685 M^{-1} cm^{-1}, respectively. The extinction coefficients of free and bound MPE·Mg(II) and MPE·Ni(II) are the same as that of MPE at 488 nm.

DNA Unwinding Angle. Each experiment contained 500 μM (DNA bp) PM2 plasmid DNA in 10 μL of buffer (50 mM Tris-HCl, 50 mM KCl, 10 mM $MgCl_2$, pH 7.5) and MPE·Mg(II) in the concentration indicated. Bovine serum albumin (1 μL) (0.5 mg/mL) was added followed by 10 units of topoisomerase I. The reaction was incubated at 37 °C for 4 h, 10 more units of enzyme was added, and the reaction proceeded for 4 more h. A 1-μL aliquot of 10% sodium dodecyl sulfate was added, and the mixture was phenol extracted twice, ether extracted twice, and then passed through a 1 mm × 5 mm column of Dowex 50W-X4 to remove MPE. The column was washed with 1 M sodium acetate, and the DNA was ethanol precipitated from the eluent. The pellet was washed with 95% ethanol, dried in vacuo, and taken up in gel loading solution (5% ficoll, 0.025% bromophenol blue, 0.025% xylene cyanol in electrophoresis buffer). The samples were loaded

onto a 1% agarose slab gel and electrophoresed for 15 h at 60 V (running buffer 40 mM Tris-HCl, 5 mM sodium acetate, 1 mM EDTA, pH 7.8). The gel was stained with ethidium bromide and photographed under UV illumination with Polaroid Type 55 film. Shifts in band position were evaluated from densitometer tracings.

Reaction Conditions for DNA Cleavage. Reactions using supercoiled pBR322 plasmid DNA (form I) as a substrate for MPE cleavage were performed in 10 μL of 10 mM Tris-HCl/50 mM NaCl, pH 7.4, unless otherwise noted. The DNA concentration was 10 μM in DNA bp unless otherwise noted. An MPE solution at 5 mM concentrations was premixed with a metal ion solution (5 mM) in a 1:1 complex followed immediately by dilution to the desired concentration. Reducing agents such as dithiothreitol and ascorbate that enhance the cleavage efficiency are always added last. Other additions are noted in the figure legends. The reaction was allowed to proceed for 60 min (unless otherwise noted) at room temperature and then immediately analyzed by agarose gel electrophoresis.

Reactions for HPLC analysis contained 20 mM sodium phosphate, pH 7.2, 950 μM (DNA bp) sonicated calf thymus DNA, 50 μM (DNA bp) bacteriophage λ [^{3}H]DNA, MPE or bleomycin, $Fe(NH_4)_2(SO_4)_2$ or $Fe(NH_4)(SO_4)_2$, and, when present, dithiothreitol (DTT) or H_2O_2 in the amounts noted in the figure legends. Bleomycin ($\epsilon_{292} = 1.45 \times 10^4$ M^{-1} cm^{-1}) (Dabrowiak et al., 1978) and MPE ($\epsilon_{488} = 5994$ M^{-1} cm^{-1}) were standardized optically prior to use. The mixture (0.2 mL) was allowed to react at 22 °C for 30 min and the reaction was terminated by ethanol precipitation. The DNA pellet was analyzed for strand scission by denaturing gel electrophoresis. The supernate was analyzed for products by HPLC or by reaction with thiobarbituric acid (TBA). The recovery of reaction products by HPLC analyses was 95% after precipitation.

Cleavage reactions for DNA termini analyses contained ≥10^4 cpm of ^{32}P end-labeled DNA made up to a total DNA concentration of 100 μM (bp) with sonicated calf thymus DNA. The buffer was 10 mM Tris-HCl/50 mM NaCl, pH 7.4. MPE, bleomycin, or EDTA was included in the amounts indicated in the figure legends, and $Fe(NH_4)(SO_4)_2$ or $Fe(NH_4)_2(SO_4)_2$ was added. The ratio of chelator to iron was 1. When present, the reactions were initiated by the addition of dithiothreitol (DTT) or H_2O_2. All of these reagent except Fe(III) were prepared in double-distilled water within minutes of use. Fe(III) solutions were freshly prepared in 1 mM aqueous H_2SO_4. The reactions were incubated at 22 °C for 30 min, and the DNA was ethanol precipitated for high-resolution gel electrophoresis.

Quantitation of DNA Cleavage. When supercoiled DNA was used as a substrate, the mean number of single-strand scissions per DNA molecule, S, was calculated from the Poisson distribution. When only forms I and II DNA are present, this equation simplifies to $S = \ln f_I$, where f_I is the fraction of form I molecules. In those cases where the cleavage reaction proceeded to form linear DNA molecules (form III), S was calculated from $f_I + f_{II} = [1 - S(2h + 1)/(2L)]^{S/2}$, where h is the distance between nicks on opposite strands needed to produce a linear molecule (16 bp) (Freifelder & Trumbo, 1969) and L is the total number of DNA base pairs in pBR322 (4362) (Sutcliffe, 1979).

The relative amounts of forms I, II, and III DNA were analyzed by agarose gel electrophoresis (1% agarose; running buffer 40 mM Tris-HCl, 5 mM sodium acetate, 1 mM EDTA, pH 7.8) and quantitated by ethidium bromide staining and densitometry (Cary 219 spectrophotometer interfaced with an Apple Computer). The film used to photograph the gels (Polaroid Type 55) was found to have a linear response in the range of DNA quantities used. In addition, since supercoiled DNA is restricted with respect to its ability to bind to ethidium bromide, it was necessary to multiply the values obtained for form I DNA by a correction factor. This factor was determined to be 1.22 by the method of Haidle (1979).

When bacteriophage λ [^{3}H]DNA was used as a substrate, the strand scissions were quantitated by monitoring the decrease in the single-strand molecule weight. The mean number of single-strand scissions per strand, P, is related to the decrease in the single-strand molecular weight of the DNA by the relationship $M_r/M_r(\text{initial}) = 2(e^{-P} + P - 1)/P^2$ (Charlesby, 1960). This relationship uses the weight average molecular weight $M_r = nM^2/(nM)$, where n represents the number of molecules of molecular weight M.

The single-strand molecular weight of the DNA after cleavage was determined by denaturation with glyoxal/Me_2SO (Carmichael & McMaster, 1980) and electrophoresis on 1.2% agarose gels. The gels were stained with acridine orange and cut into 1-mm slices with a Hoefer SL280 gel slicer, and the radioactivity of each slice was determined. The number of molecules contained in each slice is represented by the radioactivity divided by the molecular weight of the oligonucleotides in that slice (n = cpm/M). Gel slices were soaked in 10 mL of Econofluor/Protosil, 95:5 (New England Nuclear), for 24 h prior to counting in a Beckman LS200B scintillation counter. The molecular weight represented by each slice was determined by comparing its migration distance to a calibration curve constructed from restriction fragments of known size. The log M_r vs. distance plot was linear throughout the range of molecular weights examined. The initial size of the bacteriophage DNA was taken to be 49 000 bp. These data were analyzed on an Apple computer to determine P. In addition, the molecular weight distribution curves of the degraded DNA were compared to theoretical curves by assuming a random degradation process (Freifelder & Davison, 1962).

HPLC of DNA Cleavage Products. The supernate from the reaction mixture was reduced to 20 μL in vacuo, and the entire sample was injected onto an Altex Ultrasphere ODS column. The solvent system was 10 mM potassium phosphate, pH 5.5/methanol; gradient elution was 0–10% methanol over 5 min; detection was UV absorption at 260 nm. Quantitation of the four nucleotide bases was determined by using adenosine as an internal standard, which was added to the reaction mixture prior to the ethanol precipitation. Thin-layer chromatography was done as described by Giloni et al. (1981).

TBA Assay. Aliquots from the reaction mixture were passed through a 1 mm × 5 m column of Dowex 50W-X4 to remove MPE. The solution was reacted with 2-thiobarbituric acid (TBA) as described, and the TBA adduct was quantitated at 532 nm (Waravdekar & Saslaw, 1959; Burger et al., 1980).

Analysis of DNA Termini by Gel Electrophoresis. The DNA product from the cleavage reactions was suspended in 4 μL of a pH 8.3, 100 mM Tris–borate/50% formamide loading buffer and heat denatured at 90 °C for 1 min. The samples were loaded onto a 0.4 mm thick, 40 cm long, 20% polyacrylamide, 1:20-cross-linked/50% urea gel and electrophoresed at 1200 V until the bromophenol blue tracking dye had moved 26 cm. Autoradiography was carried out at −50 °C on Kodak X-omat AR film.

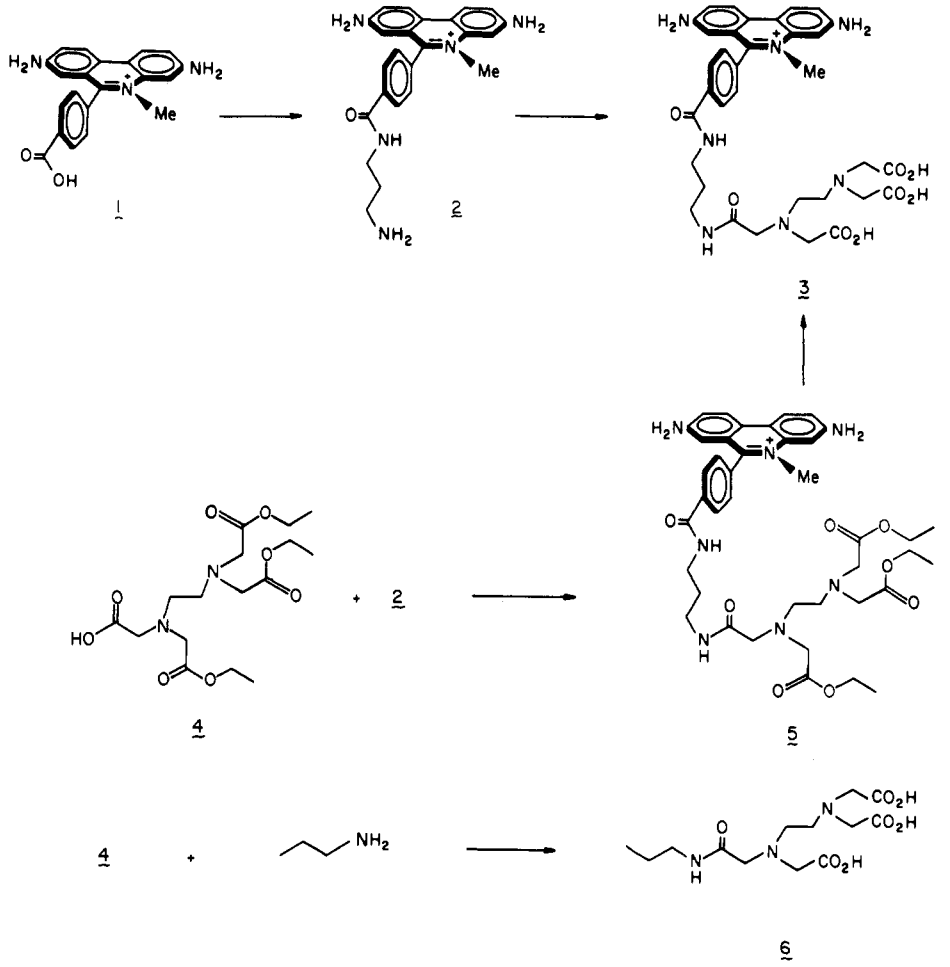


FIGURE 1: Scheme for the synthesis of methidiumpropyl-EDTA (MPE) **3**.

The presence of phosphoryl groups on the 5′-termini of DNA fragments was tested with calf intestine alkaline phosphatase. Degraded DNA was passed through a 1 mm × 5 mm column of Dowex 50W-X4 cation-exchange resin in order to remove the MPE. The [^{32}P]DNA was recovered in the void volume while the MPE remained on the column. The DNA was ethanol precipitated, dissolved in 50 μL of 40 mM Tris-HCl/5 mM NaOAc, pH 7.8, and heat denatured at 90 °C for 5 min. Calf intestinal alkaline phosphatase was added, and the sample was incubated at 37 °C for 30 min. The reaction was terminated by ethanol precipitation and taken up in loading buffer for gel electrophoresis. Similarly, DNA from a dimethyl sulfate G chemical-sequencing reaction (Maxam & Gilbert, 1977) was subjected to the same process (except for the Dowex treatment) in order to remove the 5′-phosphoryl groups.

The nature of the 3′-termini was examined by using T4 polynucleotide kinase to remove 3′-phosphoryl groups (Cameron & Uhlenbeck, 1977). DNA product from the cleavage reactions was purified on Dowex as described above to remove MPE and then ethanol precipitated. The pellet was dissolved in 20 μL of H_2O, heat denatured at 90 °C for 5 min, and chilled on ice. A total of 20 μL of a buffer containing 20 mM Tris-HCl, 20 mM magnesium chloride, pH 6.6, and 10 mM β-mercaptoethanol was added followed by 4 μL of T4 polynucleotide kinase (1.5 units/μL). The reaction was incubated at 37 °C for 1 h and ethanol precipitated for polyacrylamide gel electrophoresis.

Radioactive glycolic acid was analyzed by cellulose thin-layer chromatography as described by Giloni et al. (1981). Silica gel scrapings were eluted with 0.5 mL of 0.01 M HCl, to which was added 10 mL of Aquasol 2 (New England Nuclear). Efficiencies were determined with ^3H_2O as an internal standard.

Results and Discussion

Synthesis. MPE **3** was synthesized by two different methods (Figure 1). *p*-Carboxymethidium **1** is a known compound available in six steps from 2-aminobiphenyl (Dervan & Becker, 1978). In method A, the imidazole (Paul & Anderson, 1962) of *p*-carboxymethidium was allowed to react with an excess of 1,3-diaminopropane in Me_2SO affording methidiumpropylamine **2**. Condensation of amine **2** with excess EDTA in dry DMF at 120 °C yielded methidiumpropyl-EDTA (MPE) **3**. In method B, amine **2** was allowed to react wtih the imidazole of triethyl ester **4**, available in two steps from EDTA (Hay & Nolan, 1975), affording the triethyl ester **5**. Hydrolysis of **5** with aqueous lithium hydroxide yielded MPE, identical with that produced by method A. Propane-EDTA **6** was synthesized as a control reagent in order to compare its DNA cleavage efficiency to that of MPE. MPE **3** and **6** contain identical chelating moieties and differ only in the fact that MPE **3** contains the DNA binder methidium. The imidazole of triethyl ester **4** was condensed with propylamine in dry DMF, followed by hydrolysis with aqueous lithium hydroxide affording **6** (Figure 1).

Binding Affinity of MPE to Calf Thymus DNA. The binding of MPE **3** to DNA can be monitored by absorption spectroscopy because like ethidium bromide, a metachromatic shift results when MPE binds to double-helical nucleic acid. The λ_{max} of MPE in the free state is 488 nm, while the λ_{max} of MPE in the presence of an excess of sonicated calf thymus

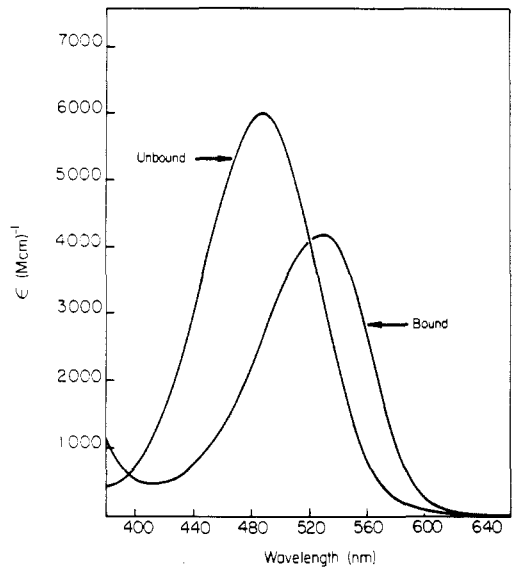


FIGURE 2: Visible spectra of MPE unbound and bound to 4 mM sonicated calf thymus DNA. The buffer was 10 mM Tris-HCl/50 mM NaCl, pH 7.4. The extinction coefficient at 488 nm for unbound MPE is 5994 M^{-1} cm^{-1} and for bound MPE is 2685 M^{-1} cm^{-1}.

Table I: Binding Affinities and Site Sizes with Calf Thymus DNA[a]

molecule	binding affinity (M^{-1})	binding site size (bp)
MPE	2.4×10^4	2
MPE·Ni(II)	1.5×10^5	2.1
MPE·Mg(II)	1.2×10^5	1.9
ethidium bromide	8.0×10^5	2.3

[a] Experimental details are described in the legend to Figure 3. The buffer was 10 mM Tris-HCl/50 mM NaCl, pH 7.4. EDTA (1 mM) was included in the metal-free MPE titration experiments.

DNA is 529 nm (Figure 2). The binding affinities were determined by absorbance titrations at 488 nm, the wavelength where the extinction coefficients of bound and unbound MPE differ most. The data are presented in Scatchard form (1949), and a comparison of the experimentally observed plots to theoretical plots generated by the binding equations of McGhee & von Hippel (1974) allows an estimation of the overall binding affinity and binding site size (Figure 3, Table I). The binding constants for the MPE·Ni(II) complex and MPE·Mg(II) complex are similar, while metal-free MPE binds with 5–6 times lower affinity. The binding site size was estimated to be approximately 2 bp for the three MPE molecules. These data indicate that MPE–metal complexes bind with similar characteristics as ethidium bromide (Table I). Similar experiments using MPE·Fe(II) or MPE·Fe(III) are not possible due to problems associated with DNA cleavage.

MPE Unwinds Superhelical DNA. Because MPE contains the DNA intercalator methidium, the unwinding of supercoiled PM2 plasmid DNA by MPE was examined by the method of electrophoretic band counting (Keller & Wendel, 1974; Wiesehahn & Hearst, 1978). Supercoiled PM2 DNA was relaxed with topoisomerase I in the presence of various amounts of MPE. Topoisomerase I requires 10 mM magnesium, and we presume we are measuring unwinding by MPE·Mg(II). MPE was removed from the superhelical DNA with cation-exchange resin, and the superhelicity of the DNA was analyzed by agarose gel electrophoresis capable of resolving topoisomers. We find that, as the concentration of MPE·Mg(II) is increased, the PM2 DNA mobility on the gel increased, indicating increased superhelicity. From the known binding constant of MPE·Mg(II) and the DNA concentration,

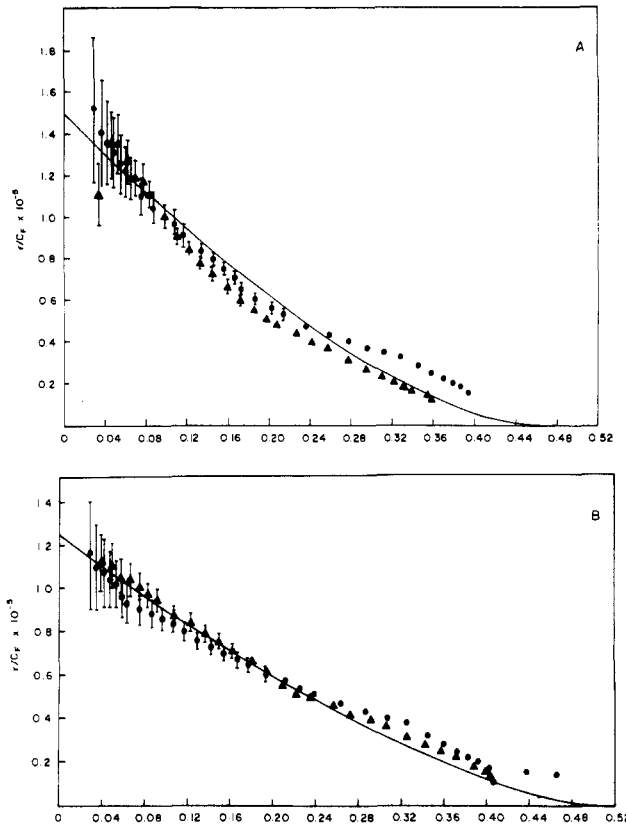


FIGURE 3: Scatchard plots were determined by spectrophotometric titrations. The results of titrations at two different DNA concentrations are shown. (A) MPE·Ni(II) was added to 9.6 μM bp (●) or 19 μM bp (▲) calf thymus DNA; (B) MPE·Mg(II) was added to 7.7 μM bp (●) or 15 μM bp (▲) calf thymus DNA. The binding density (r), concentration of bound MPE per bp, is plotted against the ratio r/C_F, where C_F is the concentration of free drug. Solid lines are theoretical plots generated by the binding equations of McGhee & von Hippel (1974) for the binding affinity (K) and binding site size (n) indicated in Table I. The binding is assumed to be noncooperative ($\omega = 1$). In data not shown, the binding affinities of (a) MPE in the presence of EDTA and (b) ethidium bromide were determined for comparison (Hertzberg, 1984).

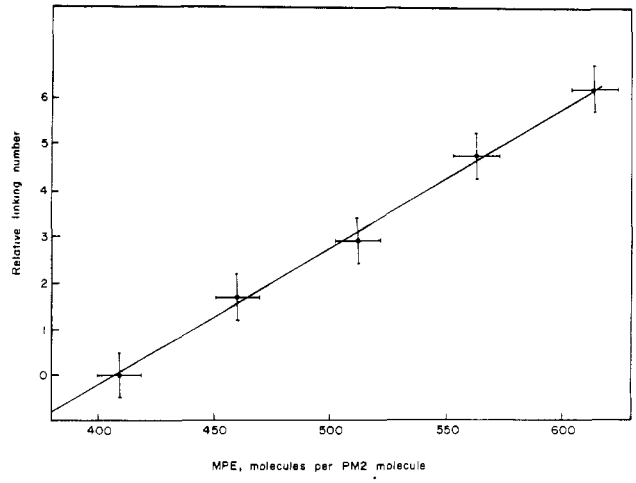


FIGURE 4: Unwinding angle plot for MPE·Mg(II) and PM2 DNA. The agarose gel was scanned with a densitometer and the band positions determined. The least-squares slope calculated from these points corresponds to 11 ± 3° unwinding per added MPE·Mg(II) molecule (Hertzberg, 1984).

the calculated ratio of bound to unbound MPE·Mg(II) (McGhee-von Hippel, 1974) is 54. A plot of the relative linking number vs. number of MPE·Mg(II) molecules gives

Table II: Cleavage of pBR322 Plasmid without Added Reducing Agents[a]

reagent	concn (μM)	form (%) I	II	III	S
EDTA·Fe(II)	100	94	6	0	0.06
6·Fe(II)	100	92	8	0	0.08
Fe(II)	100	92	8	0	0.08
MPE	1.0	93	7	0	0.07
MPE·Fe(II)	1.0	3	96	1	3.17
MPE·Fe(II)	0.1	44	56	0	0.81
MPE·Fe(II)	0.01	81	19	0	0.21

[a] Form I pBR322 DNA (10 μM bp), reagent, and buffer (10 mM Tris-HCl, 50 mM NaCl, pH 7.4) were allowed to react at 22 °C for 60 min. Forms I–III were analyzed with agarose gel electrophoresis and quantitated after ethidim bromide staining by densitometry. S = single-strand scissions per DNA molecule.

rise to an apparent unwinding angle of 11 ± 3° per bound MPE·Mg(II) molecule (Figure 4). Bresloff & Crothers (1975) found that, for ethidium bromide, 80% of bound dye is intercalated. The unwinding angle for intercalated ethidium is 26–29° (Wang, 1974; Wiesehahn & Hearst, 1978). If MPE·Mg(II) unwinds DNA 26° similar to ethidium, then 42% of MPE·Mg(II) is intercalated and 58% is outside bound.

Cleavage Efficiency of DNA by MPE·Fe(II). Cleavage efficiency was examined by following the conversion of supercoiled pBR322 plasmid DNA (form I) to open-circular (form II) and linear forms (form III). Although most DNA cleavage reactions by MPE·Fe(II) are carried out in the micromolar range, we find that premixing the MPE and metal ion solution at millimolar concentrations followed by immediate dilution to the desired micromolar concentration achieves consistent results with higher cleavage efficiencies than premixing at the more dilute micromolar concentrations used in the original report (Hertzberg & Dervan, 1982).

Control experiments shown in Table II reveal that EDTA·Fe(II), propane-EDTA–Fe(II), or Fe(II) alone at concentrations as high as 100 μM does not cleave form I plasmid DNA (10 μM in base pairs) to any significant extent (0.06 single-strand scission per plasmid). MPE at 1 μM concentration in the absence of Fe(II) does not cleave form I plasmid DNA efficiently (0.07 single-strand scission per plasmid). However, MPE·Fe(II) at 1.0 μM concentration cleaves form I plasmid DNA to 96% form II, affording 3.2 single-strand scissions per plasmid. Therefore, MPE·Fe(II) at 2 orders of magnitude lower concentration than propane-EDTA–Fe(II) cleaves DNA 50 times more efficiently (Table II). Finally, in the absence of O_2, MPE·Fe(II) does not cleave DNA (data not shown).

Reducing Agents. Although MPE in the presence of Fe(II) and O_2 is sufficient to cleave plasmid DNA, the addition of reducing agents to a solution of DNA and MPE·Fe(II) enhances the efficiency of the cleavage reaction (Hertzberg & Dervan, 1982). We find that addition of sodium ascorbate, dithiothreitol, or NADH in the 10 μM to 5 mM concentration range enhances the cleavage efficiency of DNA cleavage by MPE·Fe(II) (Figures 5 and 6). For example, in the presence of 1.0 mM dithiothreitol, propane-EDTA–Fe(II) at 1.0 μM concentration affords 0.05 single-strand scission per DNA plasmid, whereas MPE·Fe(II) at 0.01 μM concentration affords 4.2 single-strand scissions per DNA plasmid (Table III). Therefore, in the presence of reducing agents, MPE·Fe(II) at 2 orders of magnitude lower concentration (0.01 μM) than propane-EDTA–Fe(II) cleaves DNA 80 times more efficiently. Comparisons of the reducing agents reveal that the order of enhancing cleavage efficiency is sodium ascorbate > dithiothreitol > NADH (Table III) with the maximum effect in the

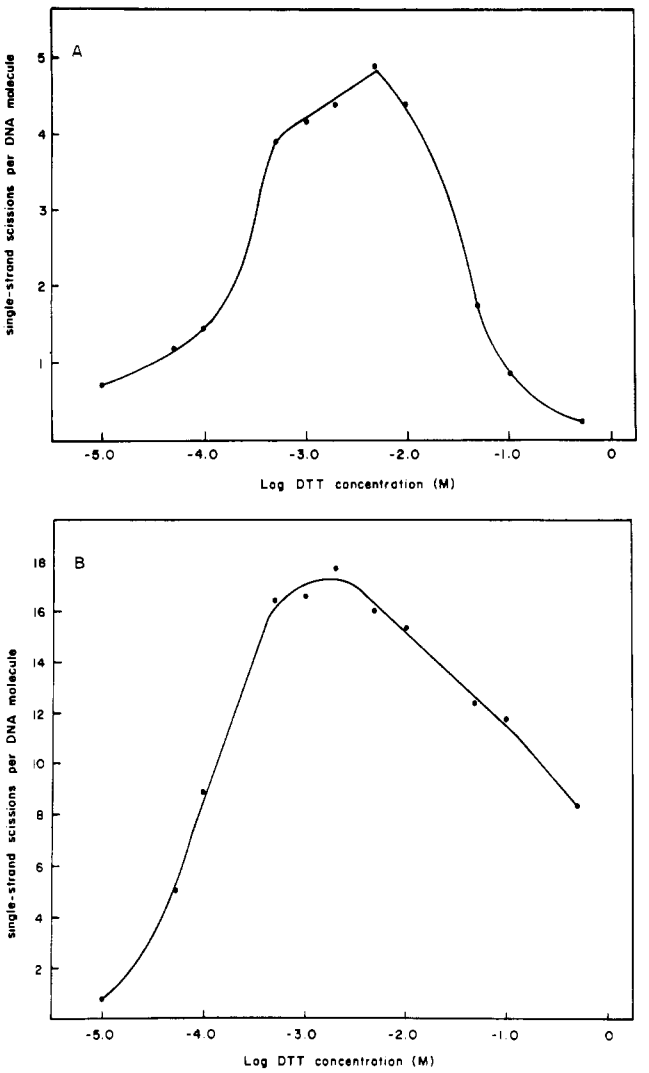
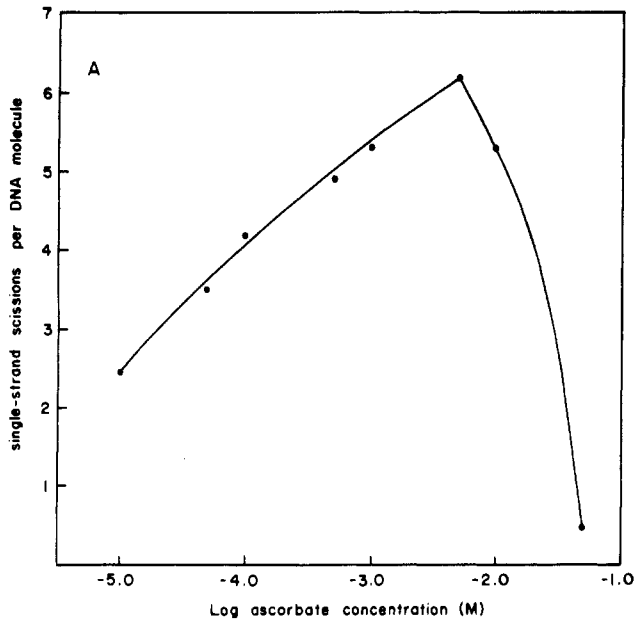


FIGURE 5: Effect of dithiothreitol (DTT) concentration on the cleavage reaction. pBR322 DNA (10 μM in bp) was incubated at 22 °C with (A) 0.01 μM MPE·Fe(II) or (B) 0.1 μM MPE·Fe(II) and the indicated concentrations of DTT for 60 min in 10 mM Tris-HCl/50 mM NaCl at pH 7.4. The number of single-strand scissions per DNA molecule was determined as described under Materials and Methods.

1–5 mM concentration range (Figures 5 and 6). At 0.01 μM concentrations of MPE·Fe(II) in the presence of 5 mM sodium ascorbate or DTT, we find that the turnover numbers (single-strand scission per MPE molecule) are 1.4 and 1.1, respectively (Figures 5 and 6). At concentrations of reducing agents greater than 1–5 mM, the cleavage reaction efficiency falls off (Figures 5 and 6).

Time. The cleavage reaction by MPE·Fe(II) in the presence of DTT was stopped at several time intervals in order to follow the rate of strand scission (Figure 7). We find that the cleavage of DNA increases linearly with time up to 200 min and then levels off. The addition of more DTT at either 60 or 120 min fails to supplement the cleavage reaction, indicating that DTT is not the limiting factor.

pH and Salt Concentration. MPE·Fe(II) was allowed to react with supercoiled DNA in the presence of 1 mM DTT over a pH range of 4–10 (Figure 8). The buffer used was a combination of phosphate, citrate, and borate, which has buffering capacity in the pH range of 4–10. We find that efficient cleavage occurs at pH 7–10 with an optimum at 8.0. At pH values below 6.0 little DNA cleavage occurs. The pH profile was examined in more detail in the pH range of 7–8.6 in a Tris buffer (data now shown). Cleavage was efficient



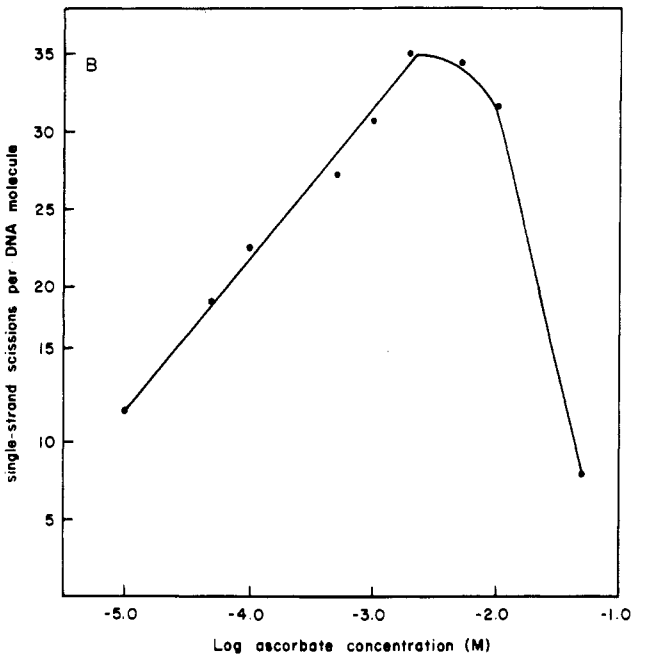


FIGURE 6: Effect of sodium ascorbate concentration on the cleavage reaction. pBR322 DNA (10 μM in bp) was incubated at 22 °C with (A) 0.01 μM MPE·Fe(II) or (B) 0.1 μM MPE·Fe(II) and the indicated concentrations of sodium ascorbate for 60 min in 10 mM Tris-HCl/50 mM NaCl at pH 7.4. The number of single-strand scissions per DNA molecule was determined as described under Materials and Methods.

throughout this range with an optimum at 7.4.

The effect of sodium ion concentration was examined (Table IV). DNA cleavage by MPE·Fe(II) in the presence of DTT (1 mM) was efficient in the range of 0.005–0.25 M NaCl. At 1 M sodium ion, the cleavage efficiency was still one-third that found for the optimum sodium ion concentration.

Metals. MPE without added Fe(II) is capable of inefficiently cleaving DNA in the presence of DTT or ascorbate, perhaps due to trace iron. The addition of Fe(II) or Fe(III) in the presence of DTT or ascorbate enhances the cleavage reaction by several orders of magnitude. Exogenous strong iron chelators such as EDTA or desferrioximine at 50 mM concentrations inhibit strand scission by MPE (Hertzberg & Dervan, 1982). In the presence of 1 mM DTT, we find that

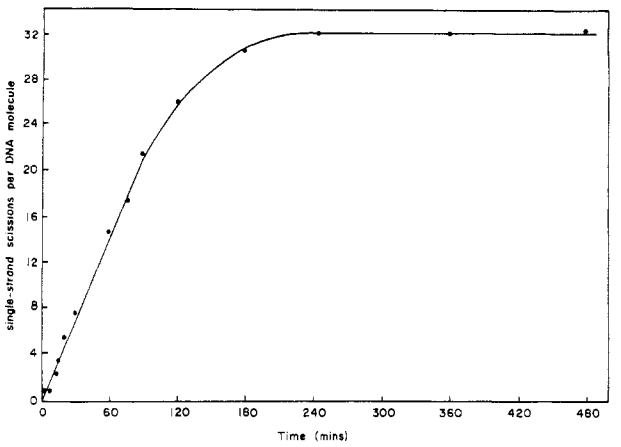


FIGURE 7: Time course of the cleavage reaction. pBR322 DNA (10 μM in bp) was incubated at 22 °C with 0.1 μM MPE·Fe(II) and 1 mM DTT in 10 mM Tris-HCl/50 mM NaCl at pH 7.4. At the indicated times, aliquots were removed, terminated with 50 mM desferrioxamine (Ciba-Geigy), and frozen in dry ice. The number of single-strand scissions per DNA molecule was determined as described under Materials and Methods.

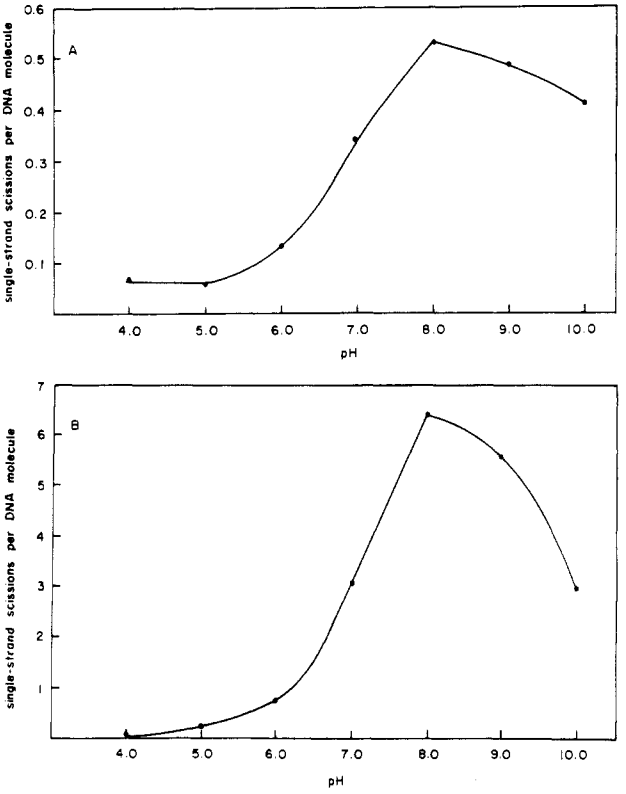


FIGURE 8: Effect of pH on the cleavage reaction. pBR322 DNA (10 μM in bp) was incubated at 22 °C with (A) 0.01 μM MPE·Fe(II) or (B) 0.1 μM MPE·Fe(II) and 1 mM DTT for 60 min. The buffer used was 20 mM citrate, 20 mM phosphate, and 10 mM borate at the indicated pH. The number of single-strand scissions per DNA molecule was determined as described under Materials and Methods.

Cu(II) and Mn(II) had little effect, while Co(II), Mg(II), Ni(II), and Zn(II) inhibited strand scission when present in stoichiometric amounts (Table V). MPE·Fe(III) in the absence of reducing agents is very inefficient at cleaving DNA. However, cleavage of DNA by MPE·Fe(III) can be slightly enhanced by the addition of millimolar concentrations of hydrogen peroxide (data not shown).

Analysis of Cleavage Products. The production of UV-absorbing, nonpolymeric products from the cleavage reaction of DNA by MPE·Fe(II) and O_2 was examined by reverse-phase HPLC. Four prominent peaks were evident in the

Table III: Cleavage of pBR322 Plasmid in the Presence of 1 mM Reducing Agents[a]

reagent	concn (μM)	reducing agent	form (%) I	II	III	S
EDTA·Fe(II)	1.0	DTT	96	4	0	0.04
EDTA·Fe(II)	1.0	ascorbate	42	58	0	0.87
EDTA·Fe(II)	10	NADH	95	5	0	0.05
6·Fe(II)	1.0	DTT	95	5	0	0.05
6·Fe(II)	1.0	ascorbate	44	56	0	0.82
6·Fe(II)	10	NADH	95	5	0	0.05
MPE·Fe(II)	0.10	DTT	0	58	42	16.6
MPE·Fe(II)	0.01	DTT	0	97	3	4.2
MPE·Fe(II)	0.10	ascorbate	0	15	85	31
MPE·Fe(II)	0.01	ascorbate	0	95	5	5.3
MPE·Fe(II)	0.10	NADH	0	95	5	5.3
MPE·Fe(II)	0.01	NADH	31	69	0	1.2

[a] Form I pBR322 DNA (10 μM bp) was incubated with the indicated concentration of reagent–Fe(II) at 22 °C in 10 mM Tris-HCl/50 mM NaCl, pH 7.4. To this was added 1 mM DTT, sodium ascorbate or NADH. The reaction was allowed to run for 60 min and analysis of forms I–III was carried out. S = single-strand scissions per DNA molecule.

Table IV: Effect of Sodium Ion Concentration on Cleavage[a]

NaCl concn (M)	form (%) I	II	III	S
0	0	88	12	8.1
0.005	0	63	37	15.4
0.01	0	64	36	15.2
0.05	0	68	32	14.1
0.10	0	70	30	13.5
0.25	0	74	26	12.5
0.50	0	91	9	6.9
1.00	0	95	5	5.2

[a] Form I pBR322 (10 μM base pairs), MPE·Fe(II) (0.1 μM), DTT (1 mM) in Tris-HCl (10 mM) buffer, pH 7.4, and the indicated NaCl concentration were allowed to react for 60 min at 22 °C.

Table V: Effect of Added Metals in the Presence of DTT on the Cleavage Reaction[a]

added metal	form (%) I	II	III	S
none	51	49	0	0.68
Fe(II)	0	58	42	16.6
Fe(III)	0	54	46	17.5
Cu(II)	64	36	0	0.45
Mn(II)	65	35	0	0.43
Co(II)	92	8	0	0.08
Mg(II)	87	13	0	0.14
Ni(II)	87	13	0	0.14
Zn(II)	82	18	0	0.19

[a] All reaction mixtures contained pBR322 DNA (10 μM in base pair), MPE (0.1 μM), metal (0.1 μM), and DTT (1 mM). Reactions proceeded at 22 °C for 60 min, followed by analysis as described under Materials and Methods. S = single-strand scissions per DNA plasmid.

chromatogram. The peaks that appear near the void volume are due to small oligonucleotides that were not removed by ethanol precipitation. The four products were identified as cytosine, guanine, thymine, and adenine (in order of elution) on the basis of retention times relative to authentic samples. These products were collected by HPLC, and their identity was confirmed by comigration with authentic standards on thin-layer chromatography. For comparison, a bleomycin–Fe(II) digest of DNA was analyzed in the same way. Eight UV-absorbing products were resolved, analogous to the eight products previously resolved by thin-layer chromatography (Giloni et al., 1981). Four products were identified as the four nucleotide bases. The four new products were collected by preparative HPLC, and each was reached with TBA to form a chromophore that displays an absorbance maximum at 532 nm. Thin-layer chromatography analysis indicated that these compounds are the base propenal derivatives of the four nucleotides as previously reported by Burger et al. (1980) and Giloni et al. (1981).[2]

A similar analysis of the DNA cleavage products by MPE·Fe(II) in the presence of DTT showed that the four DNA bases were released and in higher yield. Other products were not seen, with the exception of a peak at 14 min that was assigned by coinjection experiments to the cyclic disulfide product of DTT oxidation. For comparison, bleomycin–Fe(II) in the presence of DTT revealed an increase in the yield of free bases and an absence of base propenals. β-Mercaptoethanol has been shown to react with base propenals (Giloni et al., 1981), and this most likely occurs with DTT as well.

Stoichiometry of Base Release and Strand Scission. The quantities of the four bases released, determined by HPLC analyses, were compared with the number of single-strand scissions determined by measuring the decrease in the single-strand molecular weight of the DNA. After cleavage of DNA by MPE·Fe(II), and DNA products were ethanol precipitated, denatured with glyoxal and Me_2SO, and electrophoresed on 1.2% agarose gels next to oligonucleotide size standards. These denaturation conditions were shown not to introduce any additional strand scissions. The total number of nucleotide bases released from DNA by MPE·Fe(II) was shown to be stoichiometric with single-strand scission (Table VI). The adenine to thymine and guanine to cytosine ratios are approximately 1.0. The A+T to G+C ratio found was 1.5, which agrees with literature values for calf thymus DNA (Marshak & Vogel, 1951). This statistical release of base is consistent with the low sequence specificity of MPE·Fe(II) cleavage of ^{32}P end-labeled restriction fragments, as analyzed by high-resolution denaturing gels (Van Dyke et al., 1982; Van Dyke & Dervan, 1982, 1983ab). For comparison, we examined the amounts of the four bases released by bleomycin–Fe(II) cleavage of DNA (Table VI), as an internal check on our methods. We find accord with the work of Povirk et al. (1978) that thymine accounts for about half of the free bases. The quantity of base propenal was determined by the colored product formed after reaction with TBA. Using our assay for strand scission and the TBA assay for base propenal (Table VI), we find that DNA strand scision by bleomycin–Fe(II) is stoichiometric with base propenal production in agreement with Burger et al. (1982).

HPLC analysis has shown that MPE·Fe(II) cleavage of DNA releases little or no base propenal from DNA. We tested for the presence of a TBA reactive species. Because the optical

[2] The trivial name base propenal is used to denote products of the form 3-pyrimidin-1-yl-2-propenal and 3-purin-9-yl-2-propenal.

Table VI: Stoichiometry of DNA Strand Scission, Base Release, and TBA-Reactive Species[a]

conditions	free bases (μM) C	G	T	A	total	single-strand cleavage (μM)	A+T/G+C
500 μM MPE·Fe(II)	0.64	0.68	0.98	1.10	3.40	3.20	1.58
500 μM MPE·Fe(II)	0.77	0.79	1.19	1.31	4.06	4.12	1.60
500 μM BLM·Fe(II)	13.9	3.2	33.6	10.1	60.8	nd	2.56

conditions	TBA reactive species (μM)	single-strand cleavage (μM)
500 μM MPE·Fe(II)	0.40	3.20
25 μM BLM·Fe(II)	3.18	3.49

[a] Reaction mixtures contained 950 μM (bp) sonicated calf thymus DNA, 50 μM (bp) bacteriophage λ [^{3}H]DNA and (a) 500 μM MPE·Fe(II) or (b) 500 μM bleomycin–Fe(II). After 30 min at 22 °C, aliquots were removed and assayed for TBA-reactive species; then, the DNA was precipitated with ethanol, and the supernatant was chromatographed as described under Materials and Methods. The remaining mixture was ethanol precipitated, and the DNA pellet was analyzed for single-strand cleavage as described under Materials and Methods. Quantities of the four bases were determined by HPLC analysis of the supernatant.

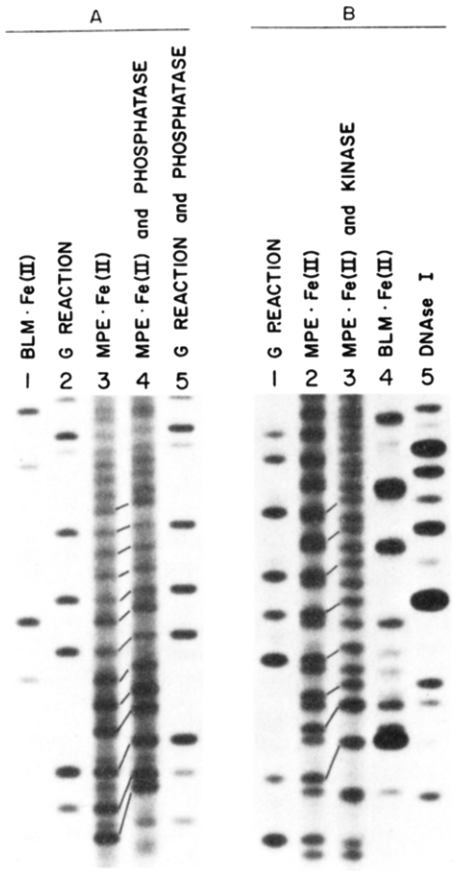


FIGURE 9: (A) Analysis of 5′-termini. The 279 base pair DNA restriction fragment labeled at the 3′-end with ^{32}P (≥10^4 cpm) and sonicated calf thymus DNA (total DNA concentration 100 μM in bp) were incubated with (lane 1) 100 μM bleomycin–Fe(II), (lane 2) G chemical-sequencing reaction (lane 3) 10 μM MPE·Fe(II) and 1 mM DTT, (lane 4) 10 μM MPE·Fe(II) and 1 mM DTT followed by calf intestinal alkaline phosphatase, and (lane 5) G chemical-sequencing reaction followed by calf intestinal alkaline phosphatase. The DNA was ethanol precipitated and electrophoresed on a denaturing 20% polyacrylamide gel as described under Materials and Methods. (B) Analysis of 3′-termini. The 381 base pair DNA restriction fragment labeled at the 5′-end with ^{32}P (≥10^4 cpm) and sonicated calf thymus DNA (total DNA concentration 100 μM in bp) were incubated with (lane 1) G chemical-sequencing reaction, (lane 2) 10 μM MPE·Fe(II) and 1 mM DTT, (lane 3) 10 μM MPE·Fe(II) and 1 mM DTT followed by T4 polynucleotide kinase, (lane 4) 100 μM bleomycin–Fe(II), and (lane 5) DNase I reaction for 1 min. The DNA was ethanol precipitated and electrophoresed on a denaturing 20% polyacrylamide gel as described under Materials and Methods.

absorbance of MPE (λ_{max} 488 nm) overlaps that of the TBA adduct (λ_{max} 532 nm), it was necessary to remove the MPE from the reaction mixture with a cation-exchange resin. Controls show that this treatment removes all MPE but not base propenals or malondialdehyde. MPE·Fe(II) treatment of DNA results in a small amount of a species that reacts with TBA to form the characteristic chromophore.

Analysis of the DNA Termini. The nature of the termini of the DNA fragments from the MPE·Fe(II) cleavage reaction was examined by comparing the electrophoretic mobility of the fragments with known DNA cleavage products on a high-resolution polyacrylamide gel. A terminal phosphoryl group increases the mobility of a DNA fragment relative to a fragment with a terminal hydroxyl group (Tapper & Clayton, 1981; Kross et al., 1982). A 279 base pair long 3′-end labeled DNA fragment was treated with MPE·Fe(II) in the presence of DTT and analyzed by denaturing 20% polyacrylamide gel electrophoresis. The mobilities of the resulting DNA fragments were compared to those produced by the reaction of bleomycin–Fe(II) on the same DNA fragment and to those produced by dimethyl sulfate treatment as in the Maxam–Gilbert sequencing protocol (Figure 9A). Both bleomycin (Kuo & Haidle, 1974) and dimethyl sulfate (Maxam & Gilbert, 1980) are known to produce 5′-termini that are phosphorylated. Figure 9A (lanes 1–3) demonstrates that MPE·Fe(II) produces oligonucleotides that comigrate with those from the bleomycin or dimethyl sulfate reactions, which suggest the presence of phosphoryl groups on the 5′-termini. Further evidence for this is obtained by treatment of these DNA products with calf intestinal alkaline phosphatase, which removes 5′-phosphoryl groups from DNA substrates (Chaconas & Van de Sande, 1980). Figure 9A (lane 5) demonstrates that phosphatase treatment of the DNA product from a dimethyl sulfate reaction results in a decrease of the electrophoretic mobility due to removal of the negatively charged groups (Kross et al., 1982). Phosphatase treatment of the DNA products from an MPE·Fe(II) cleavage reaction (Figure 9A, lane 4) produces the same shift in electrophoretic mobility, supporting the assignment that MPE·Fe(II) cleavage results in 5′-phosphoryl groups.

In order to investigate the nature of the 3′-termini, a 378 base pair DNA fragment was labeled with ^{32}P at the 5′-end. Dimethyl sulfate treatment is known to lead to phosphorylated 3′-termini (Maxam & Gilbert, 1980) while DNase leads to hydroxylated 3′-termini. The bleomycin–Fe(II) reaction produces 3-′ends that consist of glycolic acid esterified though its hydroxyl group to the phosphate termini (Giloni et al., 1981). The electrophoretic mobilities of these three types of ends can all be resolved and are illustrated in Figure 9B (lanes 1, 4, and 5). The reaction of MPE·Fe(II) with this 5′-end-labeled fragment resulted in two distinguishable DNA products in roughly equal proportions (Figure 9B, lane 2). Comparison of the mobilities of these fragments with those in lanes 1, 4,

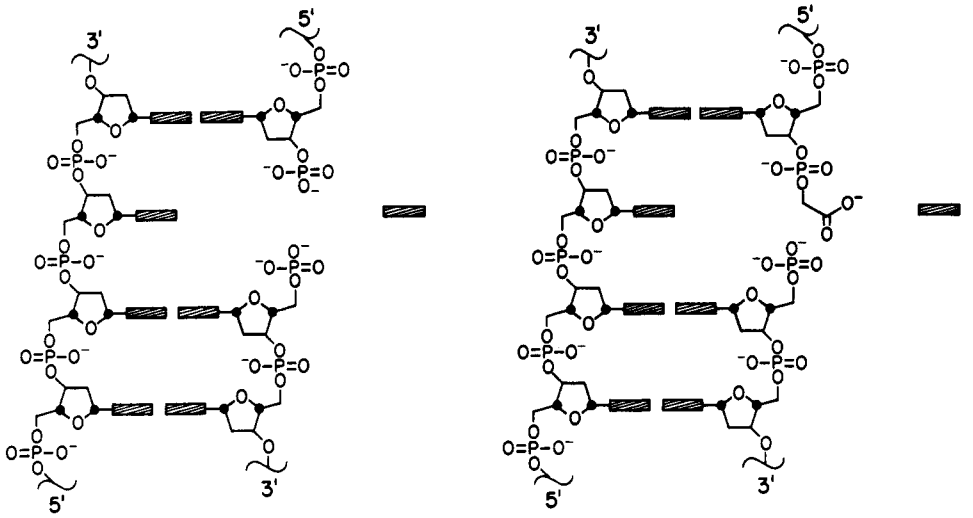


FIGURE 10: Illustration of products from MPE·Fe(II) cleavage of DNA: the DNA termini at the cleavage site are 5′-phosphate and roughly equal proportions of 3′-phosphate and 3′-(phosphoglycolic acid). Free base and not base propenal is the released product per cleavage event.

and 5 reveals that the slower moving band of each doublet comigrates with fragments produced by the dimethyl sulfate reaction, while the faster moving band comigrates with fragments produced by the bleomycin–Fe(II) cleavage reaction. This suggests that both 3′-phosphate and 3′-(phosphoglycolic acid) groups are produced by MPE·Fe(II) cleavage of DNA in roughly equal proportions.

To test for the presence of 3′-phosphate groups, half of the sample obtained from MPE·Fe(II)/DTT cleavage was treated with T4 polynucleotide kinase in the absence of ATP or ADP. T4 polynucleotide kinase has been shown to be effective as a 3′-phosphatase under these conditions (Cameron & Uhlenbeck, 1977). Comparison of lanes 2 and 3 in Figure 9B illustrates that one of the bands of each doublet produced by MPE-Fe(II) cleavage disappears upon kinase treatment and a new band appears. The bands that disappear comigrate with oligonucleotides produced in the dimethyl sulfate reaction (with 3′-phosphoryl termini) while the new bands that appear comigrate with oligonucleotides produced in the DNase reaction (3′-hydroxyl termini). These results support the assignment that the slower moving band of each doublet is a DNA fragment with a 3′-phosphoryl group.

The faster moving band of each doublet in lane 2 is resistant to T4 polynucleotide kinase. This band appears to comigrate with fragments produced by bleomycin–Fe(II). The 3′-terminus of the bleomycin-induced scission is also resistant to kinase treatment (Kross et al., 1982). These results suggest that MPE·Fe(II) scission might produce some DNA products with phosphoglycolic acid groups at their 3′-termini. To test this, DNA labeled with ^3H at the 5′-position was allowed to react with MPE·Fe(II) in the presence of DTT, ethanol precipitated, and then hydrolyzed in 6 N HCl (2 h, 110 °C). The products were treated with alkaline phosphatase to release any labeled glycolic acid. This was analyzed by cellulose thin-layer chromatography and an ^3H-labeled product was found that comigrated with authentic glycolic acid. The product was eluted from the plate, and the TMS derivative was prepared with *N*-(trimethylsilyl)imidizole (Pierce). GC–MS analysis confirmed the product as glycolic acid. We find the amount of glycolic acid recovered is 0.35 equiv of the thymine released from the DNA (HPLC analysis of the reaction mixture supernate).

The two types of DNA products at the 3′-ends occur with MPE·Fe(II) cleavage of DNA in the presence or absence of DTT. In addition, activation of MPE with Fe(III) in the presence of H_2O_2 gives rise to the same doublets on the gel, although the relative amounts of the two bands are different. MPE·Fe(III) with H_2O_2 produces a greater percentage of 3′-phosphoryl groups than either MPE·Fe(II) or MPE·Fe(II) with DTT (data not shown). To test the possibility that the phosphoglycolic acid 3′-end was degraded by H_2O_2, a set of oligonucleotides generated by MPE·Fe(II) was purified on cation-exchange resin and subsequently treated with H_2O_2. The intensity of the bleomycin-like band did not decrease, indicating that it is stable to H_2O_2 alone.

In controls not shown, millimolar concentrations of EDTA·Fe(II) produce strand scissions in DNA. EDTA·Fe(II) in the presence or absence of DTT produces the same 3′-termini as MPE·Fe(II). Cleavage with Fe(III) and H_2O_2 gives rise to a greater percentage of 3′-phosphoryl groups. For comparison, bleomycin–Fe(II) cleavage of DNA results mostly in a 3′-(phosphoglycolic acid) terminus whether DTT is present or not. Activation with bleomycin–Fe(III)/H_2O_2 (lane 9) appears to give a similar pattern. There are minor products that appear as shadows on major bands, indicating that bleomycin produces minor amounts of 3′-termini that are not phosphoglycolic acid groups.

Mechanism of DNA Cleavage by MPE·Fe(II). The mechanism of the DNA strand scission by MPE·Fe(II) is not understood in detail. However, a few key features have emerged. From the product analyses, the correspondence of one released base per cleavage event and the nature of the DNA polymer termini [5′-phosphoryl, 3′-phosphoryl, and 3′-(phosphoglycolic acid)] suggest that the cleavage reaction is oxidative degradation of the deoxyribose ring (Figure 10). Because the cleavage products are the same from EDTA·Fe(II) and MPE·Fe(II), we believe the nature of the oxidizing species is not due to any special property of the methidium portion of MPE. Recently, the 3′-termini of DNA strand scission mediated by γ-radiolysis of DNA were determined by high-resolution denaturing gel electrophoresis and were found to consist of the same mixture of 3′-phosphoryl and 3′-(phosphoglycolic acid) groups (Henner et al., 1982, 1983). Since it is established that hydroxyl radical is responsible at least in part for DNA damage induced by ionizing radiation (Ward, 1975; Armel et al., 1977), these results support the idea that MPE·Fe(II) strand scission could be mediated by production of hydroxyl radicals near the deoxyribose in the minor groove of DNA. The DNA cleavage reaction by MPE is known to be dependent on ferrous ion and oxygen and

enhanced by addition of reducing agents such as ascorbate and dithiothreitol. These results are consistent with the iron-mediated reduction of dioxygen to hydroxyl radical. Although we present no evidence here regarding how many of the intermediate reduced oxygen species (e.g., superoxide and hydrogen peroxide) are metal bound or free, we have previously shown that the enzyme superoxide dismutase (SOD), which converts superoxide to hydrogen peroxide and oxygen, inhibits MPE·Fe(II) cleavage of DNA, indicating the apparent importance of free O_2^- (Hertzberg & Dervan, 1982). Catalase, which converts hydrogen peroxide to water and oxgen, inhibits the MPE·Fe(II) cleavage of DNA, indicating the apparent importance of free hydrogen peroxide as an intermediate in the mechanistic cascade leading to DNA strand scission (Hertzberg & Devan, 1982). The inhibition of cleavage at very high concentrations of dithiothreitol (>5 mM) is consistent with the cleaving agent being a quenchable radical species. In addition, the ultimate cleaving agent appears to be diffusible. Attachment of EDTA·Fe(II) to a sequence-specific DNA binding molecule such as distamycin affords EDTA·Fe(II)-mediated cleavage at discrete sites on DNA restriction fragments. From analysis of end-labeled restriction fragments on high-resolution denaturing gels, we find that distamycin–EDTA·Fe(II) cleaves *four* base pairs contiguous to a five-base-pair A+T-rich binding site (Schultz et al., 1982; Taylor et al., 1983; Schultz & Dervan, 1983, 1984). One explanation for this multiplet of cleavage reactions at a discrete five-base-pair binding site is that the cleaving species generated by EDTA·Fe(II) is a diffusible reactive species, consistent with the assignment of hydroxyl radical as the DNA cleaving species. In summary, the dependence of the cleavage reaction on Fe(II) and O_2, the enhancement of cleavage by reducing agents at 1 mM concentration, the inhibition of cleavage by superoxide dismutase, catalase, and very high concentrations (>5 mM) of DTT, the diffusible nature of the cleaving species, the nature of the cleavage products, the correspondence of one base release per strand scission, and the agreement of the DNA cleavage products with cleavage of DNA by γ-radiolyses support the tentative assignment of hydroxyl radical as the DNA cleaving species.

Finally, the dissimilarities in the DNA cleavage products (free base vs. base propenal, two vs. one major 3′-terminus) indicate that MPE·Fe(II) and BLM·Fe(II) do not share a major common oxidative pathway for cleavage of DNA. If DNA cleavage by MPE·Fe(II) is hydroxyl radical mediated, then one might conclude that free hydroxyl radical is now excluded as the major mechanistic pathway for BLM·Fe(II)-induced cleavage of DNA.

Summary

Methidiumpropyl-EDTA (MPE) has been synthesized by two independent routes from *p*-carboxymethidium. MPE·Mg(II) binds to calf thymus DNA with an overall binding constant of 1.2×10^5 M^{-1} (50 mM Na^+, pH 7.4) and a binding site size of two base pairs. MPE·Mg(II) unwinds DNA 11 ± 3°. MPE·Fe(II) in the presence of oxygen cleaves DNA efficiently and with low sequence specificity. Reducing agents such as sodium ascorbate and dithiothreitol enhance the efficiency of the cleavage reaction substantially. Optimum conditions for cleavage of supercoiled DNA (10 μM in base pairs) are MPE·Fe(II) at 0.01 μM concentration followed by addition of sodium ascorbate at 5 mM concentration (or dithiothreitol at 5 mM) in a buffer of 10 mM Tris-HCl/50 mM NaCl, pH 7.4, at 22 °C for >3 h. The number of strand scissions on form I DNA per MPE·Fe(II) molecule is greater than one. Metals such as Co(II), Mg(II), Ni(II), and Zn(II) inhibit strand scission by MPE. Exogenous strong ion chelators such as EDTA or desferrioximine (50 mM concentrations) inhibit strand scission by MPE. The enzymes superoxide dismutase and catalase inhibit strand scission by MPE. High concentrations (>5 mM) of dithiothreitol inhibit strand scission. The products from DNA cleavage by MPE·Fe(II) in the presence or absence of reducing agents are the four nucleotide bases, 5′-phosphate, and roughly equal proportions of 3′-phosphate and 3′-(phosphoglycolic acid) termini. These products are consistent with oxidative degradation of the deoxyribose ring of the DNA backbone, most likely by hydroxyl radical.

Because MPE·Fe(II) is a useful DNA cleaving reagent for the determination of binding sites of small molecules on native DNA and in the study of chromatin structure, the results presented here bear on the interpretation and the utility of these techniques. The cleavage inhibition patterns observed on high-resolution denaturing gels from MPE·Fe(II) cleavage of small molecule protected DNA restriction fragments [MPE·Fe(II) footprinting] are most likely the result of a steric blockade to MPE·Fe(II) binding. The binding affinity of MPE·Mg(II) may be useful for estimating minimum binding affinities from the footprinting method for small molecules bound at specific sites on native DNA. The characterization of the DNA product termini produced by MPE·Fe(II) cleavage is helpful for interpreting DNA cleavage inhibition patterns on high-resolution denaturing gels. Finally, the optimization of the DNA cleavage reaction conditions allows the use of minimum quantities of MPE·Fe(II) in footprinting studies, which in turn minimizes MPE·Fe(II)-induced perturbations on the DNA.

Registry No. **1**, 66442-94-2; **2**·Cl^-, 90900-11-1; **3**·Cl^-·K, 90900-12-2; **3**·Cl^-, 90912-87-1; **4**, 90359-20-9; **5**·Cl^-, 90900-13-3; **6**, 90900-14-4; **6**·Fe(II), 90912-88-2; MPE·Ni(II), 90900-15-5; MPE·Mg(II), 90900-16-6; MPE·Fe(II), 83789-87-1; EDTA·Fe(II), 15275-07-7; EDTA, 60-00-4; O_2, 7782-44-7; Fe, 7439-89-6; Co, 7440-48-4; Mg, 7439-95-4; Ni, 7440-02-0; Zn, 7440-66-6; *N*-ethylmorpholine, 100-74-3; propylamine, 107-10-8.

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