Copper-Dependent Cleavage of DNA by Bleomycin†

Guy M. Ehrenfeld,‡,§ Joshua B. Shipley,‡ David C. Heimbrook,§ Hiroshi Sugiyama,‡ Eric C. Long,‡ Jacques H. van Boom,∥ Gijs A. van der Marel,∥ Norman J. Oppenheimer,⊥ and Sidney M. Hecht*,‡,§

Departments of Chemistry and Biology, University of Virginia, Charlottesville, Virginia 22901, Chemical Research and Development, Smith Kline & French Laboratories, Swedeland, Pennsylvania 19479, Department of Organic Chemistry, University of Leiden, Leiden, The Netherlands, and Department of Pharmaceutical Chemistry, University of California, San Francisco, California 94143

Received July 8, 1986; Revised Manuscript Received September 24, 1986

ABSTRACT: DNA strand scission by bleomycin in the presence of Cu and Fe was further characterized. It was found that DNA degradation occurred readily upon admixture of Cu(I) or Cu(II) + dithiothreitol + bleomycin, but only where the order of addition precluded initial formation of Cu(II)–bleomycin or where sufficient time was permitted for reduction of the formed Cu(II)–bleomycin to Cu(I)–bleomycin. DNA strand scission mediated by Cu + dithiothreitol + bleomycin was inhibited by the copper-selective agent bathocuproine when the experiment was carried out under conditions consistent with Cu chelation by bathocuproine on the time scale of the experiment. Remarkably, it was found that the extent of DNA degradation obtained with bleomycin in the presence of Fe and Cu was greater than that obtained with either metal ion alone. A comparison of the sequence selectivity of bleomycin in the presence of Cu and Fe using ^{32}P-end-labeled DNA duplexes as substrates revealed significant differences in sites of DNA cleavage and in the extent of cleavage at sites shared in common. For deglycobleomycin and decarbamoylbleomycin, whose metal ligation is believed to differ from that of bleomycin itself, it was found that the relative extents of DNA cleavage in the presence of Cu were not in the same order as those obtained in the presence of Fe. The bleomycin-mediated oxygenation products derived from *cis*-stilbene were found to differ in type and amount in the presence of added Cu vs. added Fe. Interestingly, while product formation from *cis*-stilbene was decreased when excess Fe was added to a reaction mixture containing 1:1 Fe(III) and bleomycin, the extent of product formation was enhanced almost 4-fold in reactions that contained 5:1, as compared to 1:1, Cu and bleomycin. The results of these experiments are entirely consistent with the work of Sugiura [Sugiura, Y. (1979) *Biochem. Biophys. Res. Commun. 90*, 375–383], who first demonstrated the generation of reactive oxygen species upon admixture of O_2 and Cu(I)–bleomycin.

Bleomycin (BLM)[1] is a glycopeptide-derived antineoplastic agent capable of effecting DNA strand scission (Hecht, 1979). In addition to a source of oxygen, bleomycin-mediated DNA degradation has been shown to require a metal cofactor (Sausville et al., 1978a,b). A description of the structure and behavior of metallobleomycin complexes is central to an understanding of the way in which bleomycin behaves as a DNA-interactive molecule and antineoplastic agent. While it has been shown that the iron (Sausville et al., 1978a), cobalt (Sugiura, 1980; Chang & Meares, 1982, 1984; Albertini & Garnier-Suillerot, 1982b), manganese (Ehrenfeld et al., 1984; Burger et al., 1984; Suzuki et al., 1985a), and vanadyl (Kuwahara et al., 1985) complexes of bleomycin are capable of mediating DNA degradation, considerable confusion has been evident regarding the properties of copper–bleomycin.

A number of interesting observations have been made concerning Cu·BLM, and these provide the motivation for its careful characterization. These have included the findings that BLM bound Cu(II) with a greater affinity than other physiologically relevant metals (Sugiura et al., 1979), that the administration of metal-free BLM was followed by Cu(II) binding in vivo (Kano et al., 1973), and that Cu(II)·BLM has potentially useful cytotoxic properties (Ishizuka et al., 1967; Crooke & Bradner, 1977; Takahashi et al., 1977; Umezawa et al., 1968; Nunn & Lunec, 1978; Rao et al., 1980). A number of structures have been suggested for Cu(II)·BLM on the basis of physicochemical studies (Iitaka et al., 1978; Takita et al., 1978; Bereman & Winkler, 1980; Solaiman et al., 1980; Dabrowiak, 1980; Antholine et al., 1984); the structure of Cu(I)·BLM has been shown to differ significantly from that of other metallobleomycin complexes (Oppenheimer et al., 1981; Ehrenfeld et al., 1985).

Cu(II)·BLM can be reduced to Cu(I)·BLM, although the facility of this process is unclear and probably dependent on the specific conditions employed (Takahashi et al., 1977; Antholine et al., 1982; Freedman et al., 1982; Kilkuskie et al., 1984). On the other hand, Cu(I)·BLM is oxidized readily to Cu(II)·BLM (Oppenheimer et al., 1981), reportedly with the generation of reactive oxygen radicals (Sugiura, 1979). Since Cu·BLM is also known to bind to DNA (Povirk et al., 1981; Oppenheimer et al., 1981), it has seemed logical to study the ability of the putative oxygen radicals to degrade DNA in analogy with the transformation mediated by other metallobleomycins.

Although initial investigation of Cu·BLM in the presence of 2-mercaptoethanol suggested that the complex could not degrade phage DNA (Shirakawa et al., 1971) and that Cu(II)

† This work was supported at the University of Virginia by NIH Research Grants CA27603 and CA38544.

* Address correspondence to this author at the Department of Chemistry, University of Virginia.

‡ University of Virginia.

§ Smith Kline & French Laboratories.

∥ University of Leiden.

⊥ University of California.

[1] Abbreviations: BLM, bleomycin; DTT, dithiothreitol; EDTA, ethylenediaminetetracetic acid; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/87/0426-0931$01.50/0

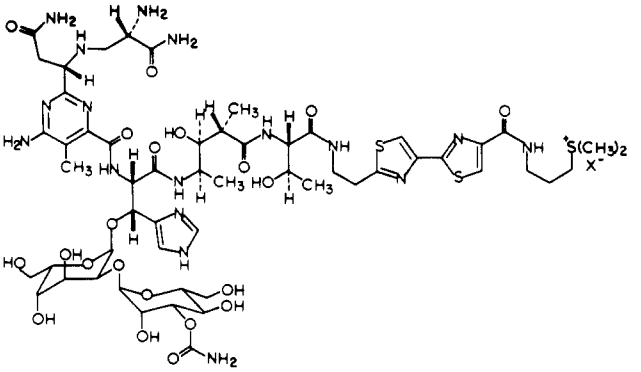
could inhibit the degradation of DNA mediated by Fe(II)·BLM (Umezawa et al., 1970; Ishida & Takahashi, 1975; Sausville et al., 1978b), studies in these laboratories subsequently demonstrated Cu(II)·BLM-mediated DNA strand scission in an C_6H_5IO-dependent reaction that required no added reducing agent (Murugesan et al., 1982; Ehrenfeld et al., 1985). Iodosobenzene-activated Cu(II)·BLM was also shown to transfer oxygen to olefinic substrates, producing oxygenated substrates qualitatively and quantitatively different than those obtained by using Fe(III)·BLM + C_6H_5IO (Murugesan & Hecht, 1985). Further studies suggested that Cu·BLM, in the presence of oxygen and an external reducing agent such as dithiothreitol or sodium dithionite, could also effect DNA strand scission (Ehrenfeld et al., 1985).

In contrast to these reports, Suzuki et al. (1985b) have recently asserted that Cu·BLM has no significant DNA cleavage activity; the observed activity in earlier reports was attributed to adventitious Fe. In an effort to reconcile these apparent differences in experimental observations, and to explore further the factors that led to earlier reports of Cu·BLM inactivity, we have reinvestigated the behavior of Cu·BLM. Reported herein are conditions optimal for the activation of Cu(I)·BLM for DNA degradation, the demonstration of bathocuproine sensitivity for the Cu·BLM-mediated process, and an analysis of the effects on DNA degradation noted for deferoxamine. Also described for the first time is the potentiation of DNA strand scission by bleomycin when both Cu and Fe were present, relative to that obtained with either metal ion alone. Characterization of DNA strand scission by Cu·BLM also included an analysis of qualitative and quantitative differences in strand selectivity of cleavage of ^{32}P-end-labeled DNA duplexes, differences in the timing of metallobleomycin activation, and the effect of metal ligand alterations on the ability of single congeners of bleomycin to mediate DNA strand scission in the presence of Cu and Fe. Additionally, this report outlines clear differences in the nature and amounts of products formed from Cu·BLM and Fe·BLM and the intriguing finding that product formation was not maximal at a 1:1 ratio of Cu and BLM.

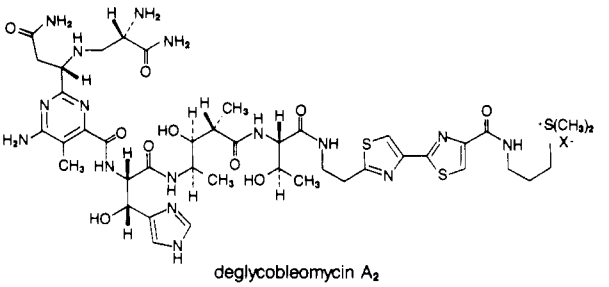
Experimental Procedures

Materials. Blenoxane was obtained from Bristol Laboratories through the courtesy of Dr. William Bradner; it was fractionated as described (Chien et al., 1977; Oppenheimer et al., 1979). Deglycobleomycin A_2 was obtained by partial hydrolysis of bleomycin A_2 (Muraoka et al., 1981; Oppenheimer et al., 1982) or by synthesis (Aoyagi et al., 1982). Decarbamoylbleomycin A_2 was obtained by partial hydrolysis of bleomycin A_2 by modification of the published procedure (Naganawa et al., 1977); the structure of the product was verified by total synthesis. Iso-BLM A_2 was obtained by isomerization of BLM A_2, as described (Nakayama et al., 1973). (See Chart I.) T4 polynucleotide kinase, DNA polymerase I (Klenow fragment), bacterial alkaline phosphatase, SV40 DNA, dithiothreitol, and agarose were obtained from Bethesda Research Laboratories. Deferoxamine mesylate was obtained from Ciba-Geigy Pharmaceuticals. Bathocuproinedisulfonate and calf thymus DNA were purchased from Sigma Chemical Co. *Eco*RI and *Hae*III restriction endonucleases were purchased from New England Biolabs; *Taq*I and *Rsa*I restriction endonucleases were from Boehringer Mannheim Biochemicals. Chelex-100 resin was purchased from Bio-Rad. Copper(I) chloride (99.99% pure), $Fe^{II}(NH_4)_2(SO_4)_2$, and Cu(II) chloride dihydrate were obtained from Aldrich Chemicals. The purity of these metals was determined by atomic absorption; contaminating Fe was 0.28% in $CuCl_2 \cdot 2H_2O$ and 0.13% in CuCl.

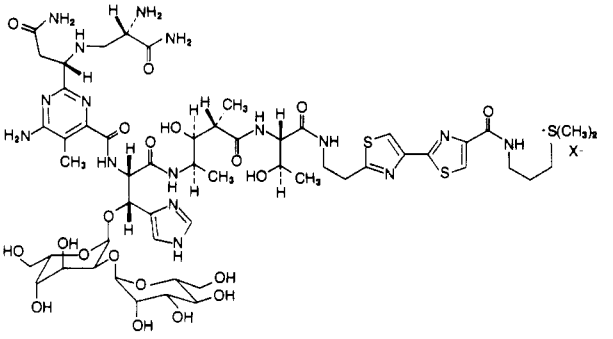
Chart I



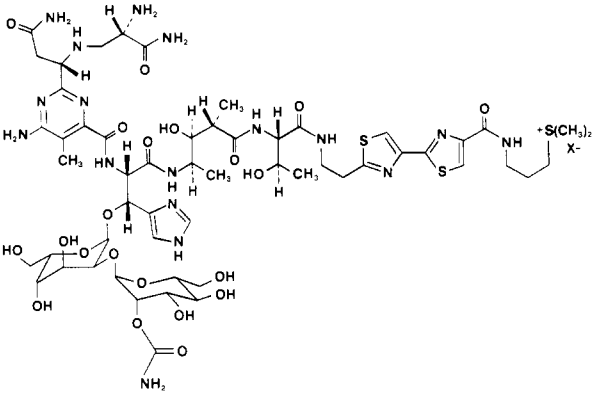
bleomycin A_2



deglycobleomycin A_2



decarbamoylbleomycin A_2



iso bleomycin A_2

Bleomycin-Mediated Cleavage of DNA in the Presence of Cu(II) and Dithiothreitol. Reaction mixtures (50-μL total volume) contained 50 mM sodium cacodylate buffer, pH 7.0, 100 μM NaEDTA, 500 ng of SV40 DNA (>95% form I), 10 μM bleomycin B_2, 40 μM DTT, 30 μM $CuCl_2$, and, where present, either 100 μM deferoxamine mesylate or 300 μM bathocuproinedisulfonate. All reaction solutions were prepared immediately prior to use with water that had been purified by passage through a Millipore-MilliQ apparatus. Further removal of adventitious metals was effected by batch treatment of solutions with portions of Chelex-100 resin (Ehrenfeld et

al., 1985). Each reaction was carried out for 10 min at room temperature. The order of addition of reaction components was as indicated in individual figure legends. Reactants were added from 10-fold concentrated stock solutions; aliquots were added to the DNA–buffer solution or premixed at 10-s intervals in the stated order prior to addition to the DNA–buffer solution. In those cases in which certain reactants were premixed separately, the reactions were initiated by combination of the preformed solutions. Following each reaction 5 μL of 75% glycerol was added to each reaction mixture, and the resulting solution was applied to a 1.2% agarose gel containing 1 μg/mL ethidium bromide. Horizontal gel electrophoresis was carried out (80 V; 3 h) in 40 mM Tris-HCl buffer, pH 7.8, containing 10 mM NaOAc and 5 mM NaEDTA. The gels were then visualized (UV light box) and photographed (Kodak no. 9 filter; Polaroid type 55 P/N film).

Preparation of DNA Restriction Fragments. SV40 DNA was digested with *Eco*RI and 3′-end-labeled with [α-^{32}P]dATP by use of the Klenow fragment of DNA polymerase I (Sanger & Coulson, 1975). The 3′-end-labeled DNA was then subjected to digestion with *Hae*III. The resulting solution was subjected to electrophoresis on a 5% nondenaturing polyacrylamide gel. Two bands (275 and 477 base pairs, respectively) were isolated by electroelution.

The 5′-end-labeled DNA employed was prepared by digesting SV40 form I DNA with *Taq*I, followed by treatment with bacterial alkaline phosphatase. The resulting DNA was labeled with [γ-^{32}P]ATP as described (Maxam & Gilbert, 1980) and then treated with *Rsa*I to yield 136- and 570-base-pair fragments, which were isolated from a 5% nondenaturing polyacrylamide gel by electroelution.

A second set of restriction fragments was prepared from SV40 form I DNA by digestion with restriction endonuclease *Bcl*I, followed by dephosphorylation of the restricted DNA with calf intestine alkaline phosphatase (Maniatis et al., 1982). The resulting linear duplex DNA was 5′-^{32}P-end-labeled as described above and then treated with *Eco*RII, which resulted in the generation of a 127-base-pair fragment and a 242-base-pair fragment. These DNA fragments were then purified by polyacrylamide electrophoresis on a 5% gel and isolated as described (Maxam & Gilbert, 1980).

Decarbamoyl-BLM and Iso-BLM-Mediated Cleavage of ^{32}P-End-Labeled DNA in the Presence of Fe(II) or Cu(II) + Dithiothreitol. Reaction mixtures (50-μL total volume) contained 50 mM sodium cacodylate, pH 7.0, sonicated calf thymus DNA (50 μM DNA nucleotide concentration), 3′-^{32}P-end-labeled DNA (~10^4 cpm), 25, 10, or 5 μM decarbamoyl-BLM A_2 or iso-BLM A_2, and 2 equiv (relative to decarbamoyl-BLM) of Fe(II) or Cu(II) + 1 mM DTT. Control reactions employed bleomycin at final concentrations of 10 and 5 μM. The reaction mixtures were demetalized as described above.

Metal ion–DTT complexes were formed first and then combined with solutions containing the BLM congener of interest. This combined solution was then added to the buffered DNA-containing solution to initiate the reaction. Reactions were maintained at 25 °C for 30 min and then treated successively with 5 μL of 3.5 M NaOAc and 2 volumes of cold ethanol. The precipitated DNA was isolated by centrifugation, washed with cold 80% ethanol, and dried. The resulting pellet was resuspended in a loading buffer consisting of 100 mM Tris–borate, pH 8.4, and 50% formamide + 0.15% bromophenol blue. The DNA was heat-denatured (90 °C, 2 min) and applied to a (0.4 × 35 × 20 cm) 8% polyacrylamide gel (1:20 cross-linked) containing 50% urea. Electrophoresis was carried out in 100 mM Tris–borate buffer, pH 8.4, containing 100 mM EDTA at 1000 V for 3 h. Autoradiography was carried out at −80 °C with an intensifying screen (Kodak X-Omat AR film).

Deglyco-BLM-Mediated Cleavage of ^{32}P-End-Labeled DNA in the Presence of Fe(II) or Cu(II) + Dithiothreitol. Reaction mixtures (30-μL total volume) contained 50 mM sodium cacodylate, pH 7.0, sonicated calf thymus DNA (15 μM DNA nucleotide concentration), 5′-^{32}P-end-labeled DNA (~10^4 cpm), 20, 10, or 5 μM deglyco-BLM A_2, and 1 equiv of Fe(II) or Cu(II) + 1 mM DTT. The reaction mixtures were initiated as described above for decarbamoyl-BLM A_2, maintained at 25 °C for 2 min, and analyzed for site-specific cleavage as described above.

Time-Dependent Activation of Bleomycin with Fe(II) + DTT or Cu(II) + DTT. To reaction mixtures (30-μL total volume) containing 20 mM solution cacodylate, pH 7.0, 40 mM NaCl, 1 mM DTT, sonicated calf thymus DNA (15 μM DNA nucleotide concentration), and 5′-^{32}P-end-labeled DNA (1.25×10^4 cpm) was added Cu(II)·BLM B_2 or Fe(II)·BLM B_2 such that the final metal ion concentration was 10 μM Cu or 5 μM Fe.

The metallobleomycins were preformed by admixture of $Fe(NH_4)_2(SO_4)_2$ or $CuCl_2$ with 1.2 equiv of bleomycin B_2. The reactions were then initiated by admixture of these preformed metallobleomycins to the reaction mixture. The reaction mixture was incubated at 25 °C, and aliquots were removed at predetermined time intervals, quenched by ethanol precipitation of the DNA, and analyzed for site-specific DNA cleavage on polyacrylamide gels as described above. Control reactions were performed in the absence of Fe(II) or Cu(II); the ability of the ethanol precipitation to quench bleomycin-mediated DNA cleavage was also verified.

Cleavage of d(CGCTTTAAAGCG) by Metallobleomycins. Reaction mixtures (50-μL total volume) contained 50 mM sodium cacodylate, pH 7.0, d(CGCTTTAAAGCG) (200 μM final nucleotide concentration), 10–20 μM BLM A_2, Fe(II) or Cu(II) at the indicated concentrations, 1 mM DTT, and deferoxamine mesylate where present. Each reaction mixture was initiated by addition of a solution containing Fe(II) or Cu(II), DTT, and deferoxamine as indicated; this solution was preincubated at 37 °C for 5 min prior to admixture to the buffered solution containing d(CGCTTTAAAGCG). The reaction mixture was incubated at 37 °C for 15 min and then analyzed promptly by HPLC on a Rainin C_{18} Microsorb (0.46 × 10 cm) column. The samples were eluted with 0.2 M ammonium formate buffer at a flow rate of 1.5 mL/min (monitored by A_{254}). The amounts of cytosine and *trans*-3-(cytosin-1-yl)propenal formed were quantified as described (Sugiyama et al., 1985a,b).

Analysis of DNA Cleavage Gels. The autoradiograms were scanned with an LKB 2202 laser densitometer interfaced to a Hewlett-Packard 3390 integrator. The data are reported as fractional cleavage, normalized over the scanned segment, with a correction factor subtracted to compensate for background absorbance of the film.

Spectrophotometric Determination of Cu(II)–Bleomycin in the Presence of Bathocuproine. To a 1-mL aerobic solution containing 50 mM sodium cacodylate buffer, pH 7.0, 80 μM $CuCl_2$, and 100 μM bleomycin A_2 was added 20 μL of 2 mM bathocuproine. The combined solution was maintained at room temperature in a cuvette for 15 min, during which time the amount of Cu(II)–bleomycin was quantified by the absorption of this complex at 600 nm (ϵ_{600} = 0.1 mM^{-1} cm^{-1}) (Freedman et al., 1982).

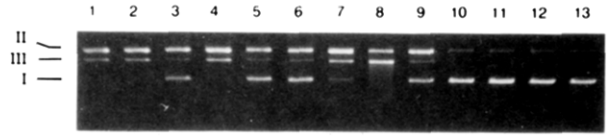


FIGURE 1: Effect of activation method on SV40 DNA strand scission by 10 μM BLM B_2 in the presence of copper and dithiothreitol. Reactions were carried out as described under Experimental Procedures. The order of addition of each reagent to the DNA solution is as indicated, and brackets indicate premixing of certain reactants prior to their (simultaneous) addition to the DNA solution: lane 1, DTT, Cu(II), BLM; lane 2, DTT, BLM, Cu(II); lane 3, BLM, Cu(II), DTT; lane 4, BLM, DTT, Cu(II); lane 5, Cu(II), BLM, DTT; lane 6, [Cu(II), BLM], DTT; lane 7, [Cu(II), BLM, DTT]; lane 8, [Cu(II), DTT, BLM]; lane 9, [DTT, BLM]; lane 10, [Cu(II), BLM]; lane 11, [Cu(II), DTT]; lane 12, BLM; lane 13, DNA alone.

Spectrophotometric Determination of Cu(I)–Bathocuproine in the Presence of Bleomycin. To a degassed solution (1-mL total volume) containing 50 mM sodium cacodylate, pH 7.0, 80 μM CuCl, and 200 μM bathocuproine were added two degassed 10-μL aliquots of a degassed 10 mM bleomycin A_2 solution; the addition of aliquots was separated by 3 min. Following equilibration at room temperature in a Thunberg cuvette, the amount of Cu(I)–bathocuproine was quantified by measuring the absorption of this complex at 480 nm (ϵ_{480} = 13.5 mM^{-1} cm^{-1}) (Sanchez-Rasero, 1981).

Spectrophotometric Determination of Cu(II)–Bleomycin in the Presence of Deferoxamine. To a 1-mL solution containing 50 mM sodium cacodylate buffer, pH 7.0, 100 μM $CuCl_2$, and 100 μM deferoxamine were added four degassed 2.5-μL aliquots of 10 mM bleomycin A_2 at 3-min intervals. Following each addition, the mixture was allowed to equilibrate at room temperature in a cuvette, and the absorption of Cu(II)–bleomycin was measured at 600 nm.

Indirect Spectrophotometric Determination of Cu(I)–Bleomycin in the Presence of Deferoxamine. To a degassed 1-mL solution in a Thunberg cuvette containing 50 mM sodium cacodylate, pH 7.0, 80 μM CuCl, and 100 μM bathocuproine was added 10 μL of a degassed 10 mM deferoxamine solution. Following equilibration at room temperature, the amount of Cu(I)–bathocuproine present was determined by measuring the absorbance at 480 nM. Comparison of the relative effects of deferoxamine and bleomycin on the concentration of the Cu(I)–bathocuproine complex afforded an indirect measure of the relative affinities of these ligands for Cu(I).

RESULTS

DNA Cleavage by Bleomycin in the Presence of Cu(II) and Dithiothreitol. As shown in Figure 1, the extent of DNA strand scission by BLM in the presence of $CuCl_2$ and dithiothreitol varied significantly as a function of the order of addition of reagents. While some cleavage was obtained in each incubation mixture containing BLM and DTT, presumably reflecting the presence of adventitious metal ions, maximal cleavage was obtained when $CuCl_2$ was premixed with dithiothreitol and then with BLM prior to their simultaneous addition to the buffer DNA solution (Figure 1, lane 8). Control experiments indicated that this extent of cleavage could be obtained only if BLM, $CuCl_2$, and DTT were present (cf. lanes 9–13). Of particular interest was the observation that alternative protocols for BLM activation using the same reagents were less productive. For example, the sequential addition of BLM, $CuCl_2$, and DTT to the buffered DNA solution gave no cleavage beyond that obtained in the absence of added $CuCl_2$ (cf. lanes 3 and 9). In each case, lack of $CuCl_2$-dependent BLM-mediated DNA strand scission was observed in those incubation mixtures in which Cu(II) and BLM were combined prior to the addition of a reducing agent and then added to the DNA solution without preincubation in the presence of DTT. Accordingly, all additional experiments were carried out in a fashion that permitted the activation of Cu·BLM.

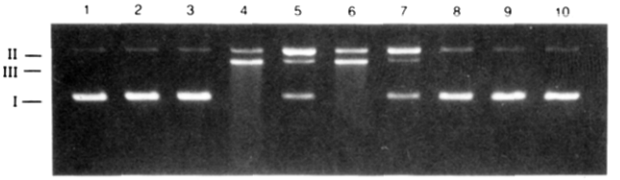


FIGURE 2: Effect of bathocuproine on BLM B_2 mediated degradation of SV40 DNA in the presence of copper and dithiothreitol. Reactions were carried out as described under Experimental Procedures. The components listed were premixed in the order given prior to their (simultaneous) addition to the buffered solution containing SV40 DNA: lane 1, Cu(II), DTT, bathocuproine; lane 2, Cu(II), bathocuproine, DTT; lane 3, Cu(II), bathocuproine; lane 4, Cu(II), DTT, bathocuproine, BLM; lane 5, Cu(II), bathocuproine, DTT, BLM; lane 6, Cu(II), DTT, BLM; lane 7, DTT, BLM; lane 8, BLM; lane 9, Cu(II), DTT; lane 10, DNA alone.

Table I: Spectroscopic Assay of Relative Binding Constants of Bleomycin, Deferoxamine, and Bathocuproine for Cu(I) and Cu(II)[a]

metal complex		apparent
I	II	K_{eq} (II/I)
Cu(II)–bleomycin A_2	Cu(II)–bathocuproine	<0.1[b]
Cu(II)–bleomycin A_2	Cu(II)–deferoxamine	<0.1[b]
Cu(I)–bleomycin A_2	Cu(I)–bathocuproine	~10 ± 2[c]
Cu(I)–bleomycin A_2	Cu(I)–deferoxamine	<0.5[c,d]

[a] The determinations were carried out as described under Experimental Procedures. Values for K_{eq} illustrate the apparent distribution of copper ion between complexes I and II under the experimental conditions employed; they do not represent true equilibrium constants. [b] Determined by monitoring [Cu(II)–bleomycin], on the basis of its absorption at 600 nm. [c] Determined by monitoring [Cu(I)–bathocuproine], on the basis of its absorption at 480 nm. [d] Determined by comparison of the equilibrium constants for Cu(I)–bleomycin ⇌ Cu(I)–bathocuproine and Cu(I)–bathocuproine ⇌ Cu(I)–deferoxamine.

The Cu dependence of BLM-mediated DNA strand scission was studied further by the use of bathocuproine, a copper-chelating agent (Smith & Wilkins, 1953). As shown in Figure 2, the extent of DNA cleavage obtained with Cu(II) + DTT + BLM (lane 6) was diminished by the addition of bathocuproine [Cu(II) + bathocuproine + DTT + BLM; lane 5] to the same level observed in the absence of added metal ion (lane 7). This observation suggested that the incremental DNA strand scission observed in lane 6, relative to that obtained in the absence of added metal ion (lane 7), was Cu-dependent. Interestingly, when the experimental protocol was changed solely by adding bathocuproine to the incubation mixture following (rather than preceding) DTT [i.e., Cu(II) + DTT + bathocuproine + BLM; lane 4], little inhibition of BLM-mediated DNA degradation was observed.

To explore further the effect of bathocuproine on BLM-mediated DNA strand scission, the relative affinities of BLM and bathocuproine for Cu(II) and Cu(I) were measured. As shown in Table I, measurement of the apparent K_{eq}'s indicated that Cu(II) bound to bleomycin at least 10 times more strongly than to bathocuproine. However, the reverse order of affinities was found for Cu(I), which bound to bathocuproine about 10 times more strongly than to bleomycin. These affinities were determined in the absence of DTT, which was employed as a reducing agent in the measurement of DNA cleavage in the presence of BLM + $CuCl_2$. Accordingly, the effect of DTT on the formation of a Cu(I)–bathocuproine complex was also measured. As illustrated in Figure 3, admixture of DTT to

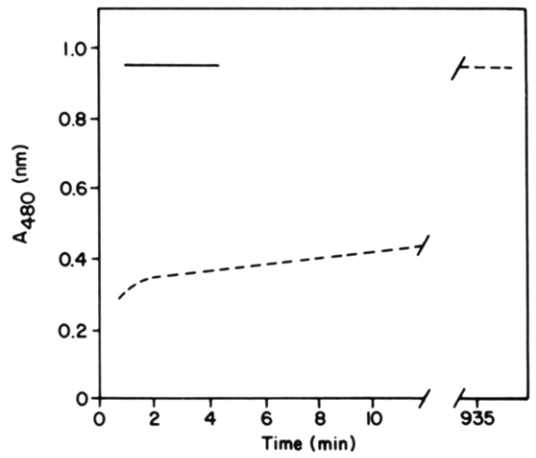


FIGURE 3: Effect of order of addition of reagents on the formation of a Cu(I)–bathocuproine complex. Two microliters of a degassed solution of 50 mM DTT was added to 1 mL of a degassed solution containing 50 mM sodium cacodylate, pH 7.0, 75 μM $CuCl_2$, and 370 μM bathocuproinedisulfonate (solid line). Alternatively, 9.25 μL of a degassed solution containing 40 mM bathocuproinedisulfonate was added to 1 mL of a degassed solution containing 50 mM sodium cacodylate, pH 7.0, 75 μM $CuCl_2$, and 100 μM DTT (dashed line).

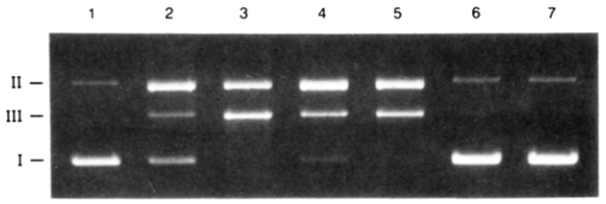


FIGURE 4: Effect of deferoxamine on BLM B_2 mediated degradation of SV40 DNA in the presence of Cu and dithiothreitol. Reactions were carried out as described under Experimental Procedures. The reaction components listed were premixed in the order given prior to their (simultaneous) addition to the buffered solution containing SV40 DNA: lane 1, BLM; lane 2, DTT, BLM; lane 3, Cu(II), DTT, BLM; lane 4, Cu(II), deferoxamine, DTT, BLM; lane 5, Cu(II), DTT, deferoxamine, BLM; lane 6, Cu(II), DTT; lane 7, DNA alone.

a preformed complex of Cu(II) and bathocuproine resulted in immediate formation of Cu(I)–bathocuproine, as judged by the appearance of a peak in the UV with λ_{max} = 480 nm (Sanchez-Rasero, 1981). However, when bathocuproine was added to a solution containing Cu(II) + DTT, formation of the same Cu(I)–bathocuproine complex occurred very slowly. In the context of the DNA cleavage experiments described above, these results suggest that the inability of bathocuproine to inhibit Cu·BLM-mediated DNA strand scission *when added subsequent to DTT* (cf. lanes 4 and 5, Figure 2) was due to the inability of bathocuproine to complex Cu(I) on the time scale (10 min) of the DNA cleavage experiment. Because bathocuproine was employed in excess in both the Cu binding and Cu·BLM-mediated DNA cleavage experiments, the presence of adventitious Fe should not affect the interpretation of the results obtained (Smith & Wilkins, 1953).

Also studied was the effect of deferoxamine, an Fe chelator (Emery, 1971), on DNA strand scission by $CuCl_2$ + BLM. As shown in Figure 4, admixture of 30 μM Cu(II), 40 μM DTT, and 10 μM BLM to a buffered solution of SV40 form I DNA resulted in significant DNA strand scission relative to that obtained in the absence of added metal ion (cf. lanes 2 and 3). Addition to the reaction mixture of 100 μM deferoxamine, either prior to or following DTT, diminished the extent of strand scission slightly [9% form I, 66% form II, and 25% form III DNA in the presence of deferoxamine (lane 4) vs. 65% form II and 35% form III in its absence (lane 3)] but did not reduce DNA degradation to the level observed in the absence of added metal ion [24% form I DNA, 63% form II DNA, and 13% form III DNA (lane 2)]. Also measured was the effect of 100 μM deferoxamine on diminishing DNA strand scission by adventitious metal ion in the presence of 10 μM BLM. Under the same conditions employed for Figure 4, incubation with BLM + deferoxamine gave 63% form I, 32% form II, and 5% form III DNA (gel electrophoresis data not shown). The modest diminution of DNA strand scission by Cu(II) + BLM + DTT observed in the presence of deferoxamine was presumably due to complexation of contaminating Fe in the $CuCl_2$ employed (0.28%, or 28 nM in this experiment).

Table II: Effect of Deferoxamine on Metallobleomycin-Mediated Cleavage of d(CGCTTTAAAGCG)[a]

metallobleomycin	deferoxamine (μM)	cytosine + 3-(cytosin-1-yl)propenal (μM)	product formation[b] (%)
Cu(II)·BLM A_2	0	6.1	100
Cu(II)·BLM A_2	2	1.4	23
Fe(II)·BLM A_2	0	6.7	100
Fe(II)·BLM A_2	2	3.9	58

[a] Reactions were carried out in the presence of 20 μM metallobleomycin and 1 mM DTT as described under Experimental Procedures. [b] Activity of each metallobleomycin in the presence of deferoxamine is expressed as a percent of the activity observed for that same metallobleomycin in the absence of deferoxamine.

Another possible aspect of deferoxamine chemistry was investigated by the use of lower concentrations of this reagent in the presence of Fe·BLM or Cu·BLM and the dodecanucleotide d(CGCTTTAAAGCG). The course of oligomer degradation was monitored by measuring the appearance of cytosine and cytosinylpropenal [3-(cytosin-1-yl)propenal] (Sugiyama et al., 1985; Murugesan et al., 1985). As shown in Table II, the yield of cytosine + cytosinylpropenal formed by 20 μM Cu(II)·BLM was diminished 77% when 2 μM deferoxamine was added to the incubation mixture. Similarly, a 42% reduction in total product was observed when 2 μM deferoxamine was added to a reaction mixture containing 20 μM Fe(II)·BLM and 200 μM DNA nucleotide. The extent of inhibition of DNA degradation was thus substantially out of proportion to the relative amounts of deferoxamine and metal ions present. Significantly, the absolute diminution of product formation [6.1 → 1.4 μM for Cu(II)·BLM A_2; 6.7 → 3.9 μM for Fe(II)·BLM A_2] also exceeded the amount of deferoxamine added (2 μM). Given the known stoichiometry of metal ion binding by deferoxamine [1:1 with Fe(III)] (Kazmi & McArdle, 1981), these results seem inconsistent with deferoxamine inhibition via simple metal complexation from individual metallobleomycins.

Cu/Fe Potentiation of BLM-Mediated DNA Strand Scission. In order to permit a more quantitative assessment of the contributions of Fe and Cu to bleomycin-mediated DNA strand scission, we also employed the dodecanucleotide d(CGCTTTAAAGCG) as a substrate for mixtures of Cu·BLM and Fe·BLM. When 200 μM d(CGCTTTAAAGCG) was treated with 10 μM BLM A_2 and 5 μM Cu(II), the concentration of cytosine + cytosinylpropenal formed was found to be 2.5 μM (Table III). When the analogous experiment was carried out with 10 μM BLM A_2 and 1 μM Fe(II), 0.8 μM cytosine + cytosinylpropenal was formed. Remarkably, when the same amount of BLM was employed in the presence of both 5 μM Cu(II) and 1 μM Fe(II), the amount of product formed (6.5 μM cytosine + cytosinylpropenal) was greatly in excess of what would have been predicted by simple addition of the ostensibly independent effects of the two metal ions on

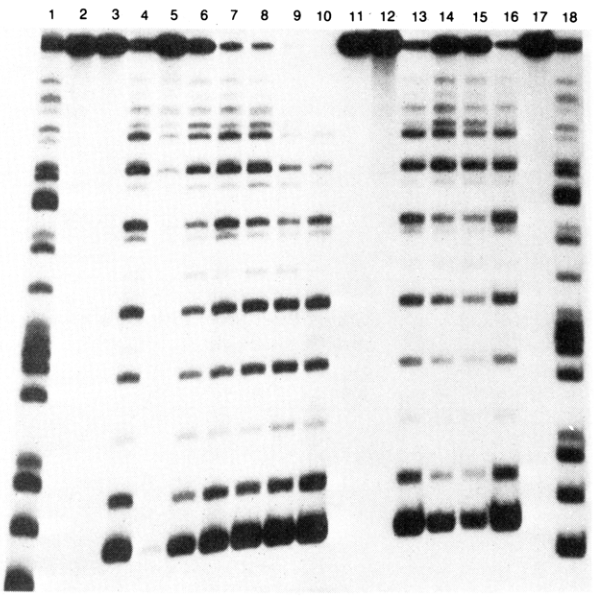


FIGURE 5: Site-specific cleavage of the 127-base-pair 5′-^{32}P-end-labeled DNA by BLM A_2 and deglyco-BLM A_2 in the presence of Fe and Cu + DTT. Reactions were carried out as described under Experimental Procedures. Reaction mixtures contained the following: lane 1, G-specific reaction; lane 2, DNA alone; lane 3, 10 μM Fe(II); lane 4, 5 μM BLM, Fe(II); lane 5, 5 μM deglyco-BLM, Fe(II); lane 6, 10 μM deglyco-BLM, Fe(II); lane 7, 5 μM BLM, Fe(II); lane 8, 5 μM deglyco-BLM, Fe(II), DTT; lane 9, 10 μM deglyco-BLM, Fe(II), DTT; lane 10, 5 μM BLM, Fe(II), DTT; lane 11, 10 μM Fe(II), DTT; lane 12, DNA alone; lane 13, 10 μM BLM, Cu(II), DTT; lane 14, 10 μM deglyco-BLM, Cu(II), DTT; lane 15, 20 μM deglyco-BLM, Cu(II), DTT; lane 16, 10 μM BLM, Cu(II), DTT; lane 17, 10 μM Cu(II), DTT; lane 18, G-specific reaction.

BLM-mediated DNA strand scission! As illustrated in the table, even relatively minor amounts of Fe gave significant potentiation of Cu·BLM-mediated DNA degradation. Since both the $CuCl_2$ and $Fe(NH_4)_2(SO_4)_2$ employed for these experiments were each contaminated with the other metal ion, the independent effect of each could not be measured directly by this technique.

DNA Cleavage of Decarbamoyl-BLM, Iso-BLM, and Deglyco-BLM. Also studied was the cleavage of DNA by three analogues of bleomycin in the presence of Fe and Cu. At least two of these three analogues are believed to have metal coordination geometries different than those of bleomycin itself (Oppenheimer et al., 1982; Sugiyama et al., 1985a, 1986). As illustrated in Figure 5, deglycobleomycin A_2 could be activated for DNA strand scission in the presence of Fe(II), Fe(II) + DTT, or Cu(II) + DTT. Both in the absence (lanes 4–7) and in the presence (lanes 8–10) of dithiothreitol, approximately twice as much Fe·deglyco-BLM A_2 was required to obtain DNA cleavage to the same extent as that mediated by Fe·BLM A_2. In the presence of Cu(II) + DTT, 10 μM BLM A_2 mediated DNA strand scission to a lesser extent than that obtained with Fe(II) + DTT, albeit not dramatically so (cf. lanes 10 and 13). DNA strand scission by Cu(II) + deglyco-BLM A_2 + DTT was less efficient than that obtained with Cu(II) + BLM A_2 + DTT at the concentrations tested (lanes 13–16), although considerable DNA cleavage was obtained.

Somewhat different results were obtained when decarbamoyl-BLM was activated in the presence of Fe and Cu (Figure 6A). At two different concentrations of the metallobleomycins, the extents of DNA cleavage obtained with Fe(II)·BLM and Fe(II)·decarbamoyl-BLM were very similar (cf. lanes 2 and 3 with lanes 7 and 8). In comparison, while 10 μM BLM mediated substantial DNA cleavage in the presence

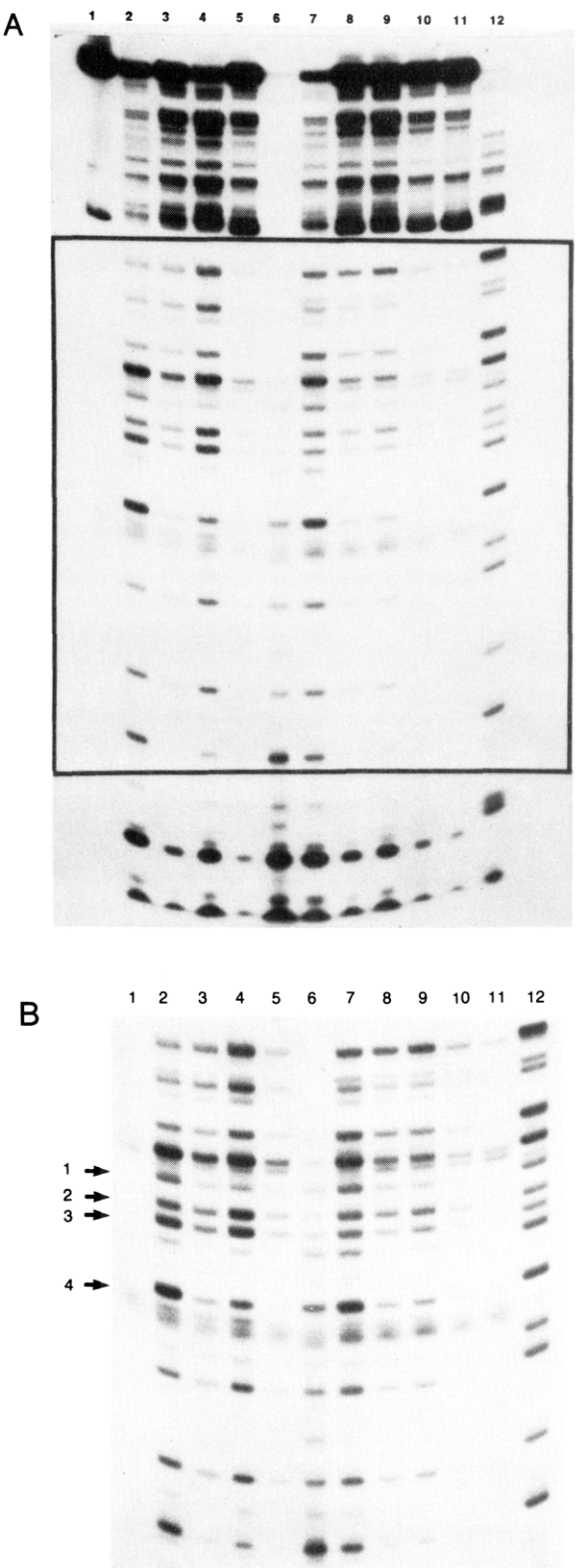


FIGURE 6: Site-specific cleavage of 3′-^{32}P-end-labeled DNA by BLM and decarbamoyl-BLM in the presence of Fe and Cu + DTT. Reactions were carried out as described under Experimental Procedures. Reaction mixtures contained the following: lane 1, no added reagents; lane 2, 10 μM BLM, Fe(II); lane 3, 5 μM BLM, Fe(II); lane 4, 10 μM BLM, Cu(II), DTT; lane 5, 5 μM BLM, Cu(II), DTT; lane 6, 25 μM decarbamoyl-BLM, Fe(II); lane 7, 10 μM decarbamoyl-BLM, Fe(II); lane 8, 5 μM decarbamoyl-BLM, Fe(II); lane 9, 25 μM decarbamoyl-BLM, Cu(II), DTT; lane 10, 10 μM decarbamoyl-BLM, Cu(II), DTT; lane 11, 5 μM decarbamoyl-BLM, Cu(II), DTT; lane 12, G-specific reaction. Panel B contains an expanded, intensified image of the central (enclosed) portion of panel A.

Table III: Potentiation of Cu–Bleomycin-Mediated Cleavage of d(CGCTTTAAAGCG) by Fe[a]

bleomycin A_2 (μM)	metal ion Cu(II)[b] (μM)	metal ion Fe(II) (μM)	cytosine + 3-(cytosin-1-yl)-propenal (μM)	enhancement of product formation[c] (%)
10	5	0[b]	2.5	
10	0[d]	1.00	0.8	
10	5	1.00	6.5	160
10	5	0.50	5.9	136
10	5	0.20	5.6	124
10	5	0.10	4.3	72
10	5	0.02	4.4	76

[a] Reactions were carried out in the presence of 1 mM DTT as described under Experimental Procedures. [b] Atomic absorption analysis of the $CuCl_2$ employed indicated that a 5 μM Cu(II) solution would contain ~0.014 μM Fe. [c] Expressed as percent enhancement relative to a reaction mixture containing 10 μM BLM A_2 and 5 μM Cu(II). [d] The concentration of Cu due to contamination of the added Fe(II) was 0.009 μM.

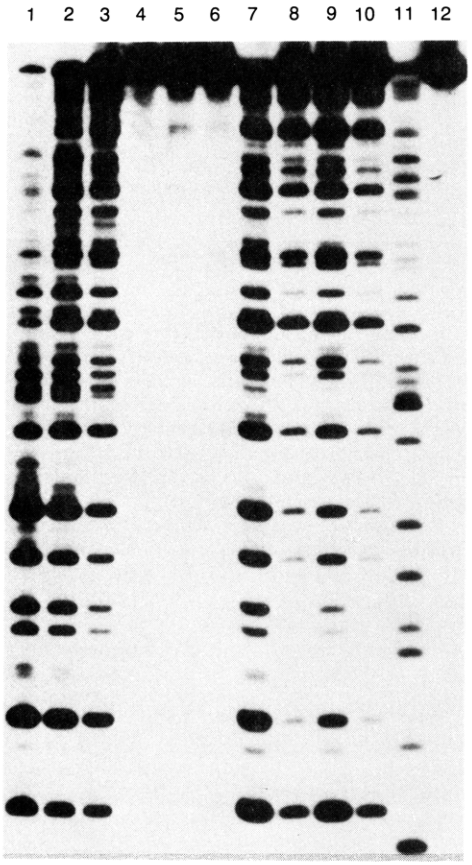


FIGURE 7: Site-specific cleavage of 3′-^{32}P-end-labeled DNA by BLM and iso-BLM in the presence of Fe and Cu + DTT. Reactions were carried out as described under Experimental Procedures. Reaction mixtures contained the following: lane 1, 25 μM iso-BLM, Fe(II); lane 2, 10 μM iso-BLM, Fe(II); lane 3, 5 μM iso-BLM, Fe(II), lane 4, 25 μM iso-BLM, Cu(II), DTT; lane 5, 10 μM iso-BLM, Cu(II), DTT; lane 6, 5 μM iso-BLM, Cu(II), DTT; lane 7, 10 μM BLM, Fe(II); lane 8, 5 μM BLM, Fe(II); lane 9, 10 μM BLM, Cu(II), DTT; lane 10, 5 μM BLM, Cu(II), DTT; lane 11, G-specific reaction; lane 12, no added reagents.

of Cu(II) + DTT (lane 4), little DNA cleavage was obtained even in the presence of 25 μM decarbamoyl-BLM + Cu(II) + DTT (lanes 9–11). Even more extreme in behavior in the presence of Fe vs. Cu was iso-BLM. As shown in Figure 7, while DNA strand scission with Fe·iso-BLM A_2 was not dissimilar to that obtained with Fe·BLM A_2, no DNA cleavage whatever was obtained when Cu(II) + DTT + iso-BLM A_2 was employed.

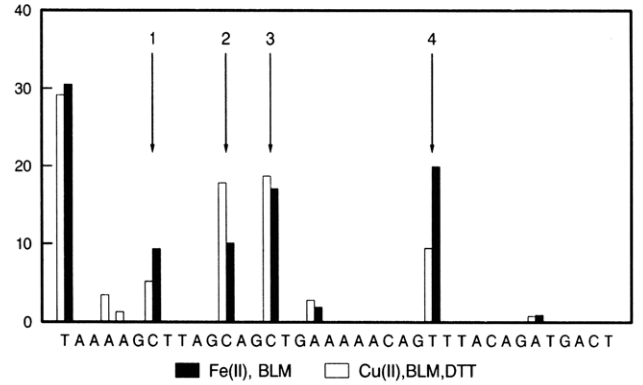


FIGURE 8: Densitometric analysis of lanes 2 and 4 from Figure 6. The numbered arrows refer to the same sites as those in Figure 6B.

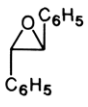
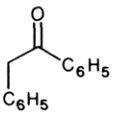
Site Specificity of DNA Cleavage by Fe·BLM and Cu·BLM. Having demonstrated DNA strand scission of 3′-^{32}P-end-labeled DNA by Cu·BLM and Fe·BLM, it was of interest to compare the DNA site specificity (D'Andrea & Haseltine, 1978; Takeshita et al., 1978; Kross et al., 1982; Mirabelli et al., 1983) for these two species. Although Fe·BLM and Cu·BLM were found to mediate cleavage largely at GC and GT sequences, it was found that the relative cleavage efficiencies at certain sites differed significantly for the two metallobleomycins (cf. lanes 2 and 3 with lanes 4 and 5 in Figure 6B). The cleavage patterns of lanes 2 and 4, for which the overall extent of DNA cleavage was quite similar, were compared by densitometric analysis, with the results represented as a histogram in Figure 8. While the extent of cleavage at site 3 was quite similar both for Fe·BLM and for Cu·BLM (17.1% and 18.7%, respectively, of total cleavage over the segment scanned),[2] considerable variation was seen at the other sites of cleavage. At site 2, for example, 17.8% cleavage was obtained with Cu·BLM but only 10.1% cleavage was observed for Fe·BLM; in comparison, the extent of cleavage by Fe·BLM was significantly in excess of that observed for Cu·BLM at sites 1 (9.4% vs. 5.2%) and 4 (20.0% vs. 9.4%). That the variation in extent of cleavage observed in Figure 6 at specific sites was not due to an inherent intraexperiment variability was established via densitometric analysis of replicate reaction mixtures, each containing 10 μM Fe(II)·BLM and 3′-^{32}P-end-labeled DNA [supplementary material (see paragraph at end of paper regarding supplementary material)]. Also established was the lack of effect of dithiothreitol on the extent of DNA cleavage at specific sites by Fe·BLM (supplementary material).

Certain DNA duplexes were found to reflect greater differences in site specificity when cleaved with Fe·BLM and Cu-BLM. This is illustrated in Figure 9 for the 5′-^{32}P-end-labeled, 127-base-pair DNA restriction fragment. As shown in the figure, a number of obvious qualitative differences in site specificity were apparent when this DNA duplex was cleaved with the two metallobleomycins; these differences were found to be reproducible in additional experiments involving this DNA fragment. Thus, the site specificity of DNA cleavage by Cu·BLM was both quantitatively and qualitatively different than that obtained with Fe·BLM.

Time-Dependent Activation of Metallobleomycins. Another qualitative difference between Fe·BLM and Cu·BLM involved the time course of activation of these two metallobleomycins. As shown in Figure 10, admixture of preformed Fe(II)·BLM

[2] The total amount of DNA degradation obtained in lane 2 and that in lane 4 were nearly identical.

Table IV: Oxidation of *cis*-Stilbene with Metallobleomycin + C_6H_5IO[a]

BLM (μmol)	metal ion		 (μmol)	 (μmol)	C_6H_5CHO (μmol)	 (μmol)	yield[b] (%)
	Cu(II) (μmol)	Fe(III) (μmol)					
2.76	2.76		0.10	0.40	0.10		22
2.76	13.8		0.20	2.0	0.10		83
	13.8		*c*		0.30		
2.76		2.76	0.05	1.3	0.10	2.0	125
2.76		13.8	0.03	0.80	0.10	1.5	88
		13.8		*c*	0.03		

[a] Reaction mixtures containing 80 mg (0.44 mmol) of *cis*-stilbene, 2.76 μmol of blenoxane, and 1 or 5 equiv of $Fe(ClO_4)_3$ or $CuCl_2$ in 3 mL of CH_3OH were treated dropwise with 15 mg (68 μmol) of iodosobenzene in 1.5 mL of CH_3OH over a period of 15–20 min. The combined solution was maintained under argon at 25 °C for 2 h. The reaction mixture was then diluted with 25 mL of water and extracted with portions of $CHCl_3$. The $CHCl_3$ extract was concentrated, and the product mixture was analyzed by HPLC on an Alltech 5-μm silica gel column. The column was washed with 15% or 30% $CHCl_3$ in cyclohexane at a flow rate of 2 mL/min (monitored by A_{254}). Products eluted at 6.0 (*trans*-stilbene oxide), 10.6 (*cis*-stilbene oxide), 27.0 (benzaldehyde), and 49.6 (deoxybenzoin) min. [b] Percent yields based on added bleomycin. [c] Trace amount of product; too little to quantitate.

FIGURE 9: Site-specific cleavage of a 127-base-pair 5′-^{32}P-end-labeled DNA by 5 μM Fe(II)·BLM A_2 (lane 1) and 10 μM Cu(II)·BLM A_2 + 1 mM DTT (lane 2). The sequence illustrated on the gel is 5′-GACTGTGAGGACTGAGGGGCCCTGAAATGAGCTTG-GGACTGTGAT····3′.

to a reaction mixture containing DNA and dithiothreitol resulted in DNA cleavage that was essentially complete at the shortest time point measured (approximately 15 s). In contrast, DNA strand scission by preformed Cu(II)·BLM was only about 25–30% complete after 5 min of incubation at 25 °C, although the extent of DNA cleavage at longer incubation times was not very different than that obtained with Fe(II)·BLM.[3]

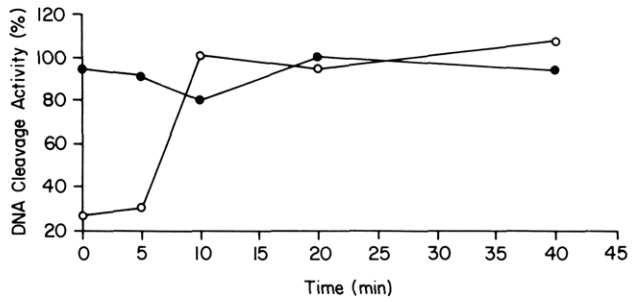


FIGURE 10: Time-dependent activation of BLM A_2 in the presence of Cu and Fe. Reactions were carried out as described under Experimental Procedures with a slight excess of BLM relative to added metal ion. DNA cleavage activity was determined by densitometric analysis of polyacrylamide gels that contained the products of digestion of 5′-^{32}P-end-labeled DNA with BLM + Fe(II) + DTT or BLM + Cu(II) + DTT. The extent of cleavage was calculated from the proportion of full-length (127-base-pair) 5′-^{32}P-end-labeled DNA converted to smaller products. The activity is expressed as the percent cleavage relative to the maximum cleavage obtained at any time point in the Fe(II)·BLM (●) and Cu(II)·BLM (○) reactions.

Oxygen Transfer from Activated Metallobleomycins to cis-Stilbene. In addition to their oxidative destruction of DNA, metallobleomycins also have been shown to transfer oxygen to low molecular weight substrates such as *cis*-stilbene in what are presumably bimolecular processes (Murugesan et al., 1982; Ehrenfeld et al., 1984; Murugesan & Hecht, 1985). This experimental system was employed for the comparison of Cu·BLM and Fe·BLM. As illustrated in Table IV, admixture of BLM + Cu(II) + C_6H_5IO in the presence of *cis*-stilbene provided *trans*-stilbene oxide, *cis*-stilbene oxide, and benzaldehyde under the experimental conditions employed. The use of a 5-fold excess of Cu(II) enhanced the formation of the stilbene oxides; the total yield of products in the presence of excess Cu(II) was about 4-fold greater than when a 1:1 Cu:BLM ratio was employed.

Repetition of the experiment under the same conditions, but with 1:1 Fe(III) and BLM, afforded benzaldehyde in amounts similar to those obtained with Cu·BLM. However, while *cis*-stilbene oxide was obtained to a greater extent than with 1:1 Cu(II)·BLM, the yield of *trans*-stilbene oxide was lower. Also formed in the presence of Fe(III)·BLM was deoxy-

[3] Because these experiments were carried out in the presence of excess bleomycin relative to metal ions, the differences in kinetics of activation cannot be attributed to competition for bleomycin molecules by adventitious metal ions.

benzoin, a product not observed with Cu(II)·BLM. In the presence of a 5-fold excess of Fe(III), the yields of *cis*- and *trans*-stilbene oxides, as well as deoxybenzoin, were *diminished* relative to those obtained with 1:1 Fe(III)·BLM. All of the products formed were bleomycin-dependent with the exception of benzaldehyde, which also formed in the absence of bleomycin. BLM enhanced benzaldehyde formation in the presence of Fe(III) but not in the presence of Cu(II).

Discussion

Our ongoing work on bleomycin activation in the presence of Cu is based on the pioneering work of Sugiura (1979), who reported that Cu(I)·BLM generated oxygen radicals from O_2, with concomitant formation of Cu(II)·BLM. Since both Cu(I)·BLM (Oppenheimer et al., 1981) and Cu(II)·BLM (Povirk et al., 1981) had been shown to bind to DNA, and oxygen radical generation in proximity to DNA has been shown to produce DNA strand scission [see, e.g., Fiel et al. (1982), Henichart et al. (1982), Lown and Joshua (1982), Aft and Mueller (1983), Suzuki et al. (1983), Hashimoto et al. (1984), Eliot et al. (1984), Hertzberg and Dervan (1984), Taylor et al. (1984), Chu and Orgel (1985), and Dreyer and Dervan (1985)], it seemed logical to expect that Cu(I)·BLM would mediate oxygen-dependent DNA degradation. In fact, DNA strand scission by Cu in the presence of other chelators is well documented (Sigman et al., 1979; Downey et al., 1980; Graham et al., 1980; Que et al., 1980; Marshall et al., 1981; Reich et al., 1981; Jessee et al., 1982; Pope et al., 1982; Uesugi et al., 1982; Aft & Mueller, 1983; Wong et al., 1984a,b; Goldstein & Czapski, 1986).

In previous work we demonstrated that DNA strand scission was obtained following activation of bleomycin in the presence of Cu(I) or Cu(II) + O_2 and a reducing agent such as dithiothreitol or dithionite (Ehrenfeld et al., 1985).[4] Bleomycin could also be activated for DNA strand scission by the use of Cu(II) + C_6H_5IO (Murugesan et al., 1982; Ehrenfeld et al., 1985) or Cu(II) + O_2 + NADPH + NADPH–cytochrome P-450 reductase (Kilkuskie et al., 1984). In each case involving dioxygen activation, DNA cleavage was maximized when experiments were carried out such that reduction of Cu(II) to Cu(I) preceded metal ion binding by bleomycin; optimal experimental protocols were provided in each case (Kilkuskie et al., 1984; Murugesan et al., 1985). These observations were consistent with earlier reports concerning the reduction of Cu(II)·BLM to Cu(I)·BLM (Takahashi et al., 1977; Antholine et al., 1982; Freedman et al., 1982). That the observed degradation of DNA by bleomycin in the presence of added Cu was not due to adventitious Fe was supported by the findings that the products of DNA strand scission in the presence of bleomycin + Cu differed from those obtained in the presence of Fe, that DNA cleavage mediated by added Cu was sometimes more extensive than that obtained with Fe, and that the amount of Fe shown to contaminate the added Cu was found to be insufficient to mediate the observed DNA damage in the presence of BLM (Kilkuskie et al., 1984; Ehrenfeld et al., 1985). Also pertinent was the finding that oxygen transfer to *cis*-stilbene mediated by BLM + Cu(II) + C_6H_5IO gave products both quantitatively and qualitatively different than those obtained with BLM + Fe(III) + C_6H_5IO (Murugesan & Hecht, 1985).

Recently, Suzuki et al (1985b) have reported that Cu(II)·BLM in the presence of dithiothreitol did not mediate significant DNA cleavage. Further, because cleavage obtained in the presence of BLM + Cu(I) + dithiothreitol was found to be inhibited by deferoxamine, but not by bathocuproine, these authors concluded that DNA strand scission in the presence of added Cu was actually due to adventitious Fe present in the added Cu. Because this paper did not specify the protocol employed for the attempted activation of bleomycin in the presence of Cu(II) + dithiothreitol and also contained implicit simplifying assumptions about the behavior of deferoxamine and bathocuproine, we have carried out additional experiments that clarify certain facets of DNA strand scission in the presence of BLM + Cu(II) + DTT.

In agreement with Suzuki et al. (1985b), we found that admixture of dithiothreitol to a preformed complex of Cu(II)·BLM B_2 resulted in no DNA strand scission during the 10-min incubation period, relative to a control that lacked added metal ion (cf. lane 9 and lanes 3, 5, and 6, Figure 1). However, admixture of Cu(II), BLM B_2, and dithiothreitol in any fashion not leading to initial formation of Cu(II)·BLM did afford considerable DNA strand scission within the 10-min incubation period (cf. lane 9 and lanes 1, 2, 4, 7, and 8, Figure 1).[5] Consistent with the measured rate of reduction of Cu(II)·BLM by alkyl thiols (Freedman et al., 1982), longer incubation times in the presence of DTT gave DNA strand scission even where preformed Cu(II)·BLM was employed (data not shown). The actual time course of activation of Cu(II)·BLM with DTT + O_2 was also measured with 5′-^{32}P-end-labeled DNA as a substrate. As shown in Figure 10, full activation in this system was achieved only after ~10 min of incubation. These results are consistent with the participation of Cu in the formation of an activated BLM complex.

Although the definition of single vs. double DNA breakage by Cu·BLM was not a specific goal of this study, the available data suggest that Cu·BLM can mediate both single- and double-strand cleavage, in common with Fe·BLM (see Figures 1, 2, and 4).

Effect of Metal-Selective Chelators on Bleomycin-Mediated DNA Strand Scission. The use of metal-selective chelating agents to study the metal dependence of DNA strand scission by bleomycin is complicated by a number of factors. These include the presence of both Fe and Cu at two different oxidation states in the incubation mixtures, the relative affinities of these four metal ions for the chelating agents and the way in which these affinities may affect the distribution of oxidation states, the presence of competing ligands (DNA, DTT, BLM) for each of the metal ions present, and the amount of time required to reach equilibrium in the presence of metal-specific chelating agents relative to the time course of DNA strand scission. Accordingly, we began our study by measuring the relative affinities of Cu(I) and Cu(II) for bathocuproine and deferoxamine.

As shown in Table I, Cu(II) was found to bind to bleomycin at least 10 times more strongly than to bathocuproine, but the reverse was true for Cu(I). Moreover, when bathocuproine was added to a solution formed by admixture of DTT and Cu(II), formation of Cu(I)–bathocuproine was found to be very slow on the time scale employed for DNA strand scission by bleomycin (Figure 3). That this was not due to rate-limiting reduction of Cu(II) → Cu(I) may be appreciated from the rapid formation of Cu(I)–bathocuproine when DTT was added to a buffered solution containing Cu(II) and bathocuproine

[4] In addition to their role in Cu(I) (re)generation, the thiols were found to be required for stabilization of Cu(I)·BLM, as the complex underwent rapid metal ion exchange (Oppenheimer et al., 1981) and free Cu(I) ions are unstable in aqueous solutions (Cotton & Wilkinson, 1972).

[5] Similar results were obtained with bleomycin A_2.

(Figure 3). Not suprisingly, therefore, admixture of bathocuproine to an incubation mixture already containing Cu(II) + DTT had no effect on DNA strand scission when BLM was added subsequently (cf. lanes 4 and 6, Figure 2). This was entirely consistent with the experimental observation made by Suzuki et al. (1985b) using the same reagents. However, when we changed the order of addition of reagents to reflect the binding rates and affinities of the chelators involved [Cu(II), bathocuproine, DTT, and then BLM; cf. Figure 3], bathocuproine reduced DNA strand scission by BLM to the same level obtained in the absence of any added metal ion (cf. lanes 5 and 7, Figure 2). Thus when run under appropriate conditions, DNA cleavage by Cu(II) + BLM + DTT was sensitive to inhibition by this copper-selective chelating agent.

Also studied was the effect of deferoxamine on DNA strand scission in the presence of Cu and BLM. As shown in Figure 4, 100 μM deferoxamine diminished the extent of DNA strand scission slightly (cf., lanes 4 and 5 vs. lane 3) but did not reduce cleavage nearly to the extent obtained in the absence of added Cu (lane 2, Figure 4). Even though deferoxamine failed to effect complete inhibition of DNA strand scission in the presence of BLM + Cu(II) + DTT, it is interesting to consider the source of the observed diminution of DNA strand scission. Possibilities include binding to deferoxamine of adventitious Fe in the incubation mixture, which could have reduced background cleavage, or direct binding of Cu(I) to deferoxamine.

A third possibility is illustrated in Table II, which demonstrates the effect of low concentrations of deferoxamine on the BLM-mediated degradation of d(CGCTTTAAAGCG), both in the presence of Cu(II) and in the presence of Fe(II). In both cases inhibition of product formation was not only out of proportion to the relative amounts of deferoxamine and metal ions present but actually greater in absolute terms than the amount of deferoxamine present. A survey of the literature provided two reported determinations of diminution of Fe·BLM-mediated DNA strand scission as a function of added deferoxamine; in one case the source of activated BLM was Fe(II) + O_2 + 2-mercaptoethanol (Sausville et al., 1978a), and in the other it was Fe(III) + H_2O_2 (Burger et al., 1984). When the published results were replotted as a linear function of deferoxamine concentration (data not shown), it became quite clear that deferoxamine also caused inhibition of Fe·BLM-mediated DNA degradation far beyond what would have been expected of a simple chelator. We suggest that for Fe·BLM, and possibly also for Cu·BLM, (metal complexes of) deferoxamine can mediate inhibition of DNA strand scission via direct destruction of the activated oxygen species. This would be analogous to the known scavenging of superoxide (Halliwell, 1985) and hydroxyl radicals (Hoe et al., 1982) by deferoxamine.

Potentiation of DNA Cleavage by Bleomycin in the Presence of Cu and Fe. One entirely novel facet of bleomycin-mediated DNA strand scission became apparent during the course of these studies. As shown in Table III, when small amounts of $Fe^{II}(NH_4)_2(SO_4)_2$ were added to incubation mixtures containing BLM A_2 + $CuCl_2$, the amount of DNA degradation observed was significantly in excess of that obtained with Cu(II) + BLM alone or with Fe(II) + BLM alone. These experiments were carried out with excess BLM, so that competition for BLM by the different types of metal ions should not have been a factor in the observed results. The molecular basis of this observation is not clear at present but could involve the redox coupling of Fe·BLM and Cu·BLM in a way that facilitated DNA degradation or, conceivably, even the binding of more than one metal ion to a single bleomycin molecule. In the context of the present study, the observation summarized in Table III has at least two important implications. The first is that since the potentiation noted in Table III is significant at levels of contaminating metal ions typically found in commercial samples of Fe and Cu, the intrinsic properties of Fe·BLM alone and Cu·BLM alone may not be accessible with such samples. The second is that the ability or inability of Cu·BLM alone to mediate DNA strand scission cannot be assessed by simple quantitation of reaction products in comparison with those obtained with Fe·BLM. Accordingly, we sought to establish criteria by which the chemistry of Cu·BLM differed *qualitatively* from that of Fe·BLM.

Effect of Metal Ligation on BLM Activation and the Specificity of DNA Cleavage. As outlined in Figures 5 and 6, for both deglyco-BLM A_2 and decarbamoyl-BLM A_2 admixture of Fe(II) in the presence of 3′-^{32}P-end-labeled DNA resulted in sequence-selective strand scission; the pattern of strand scission was quite similar to that obtained with Fe(II)·BLM A_2. The extent of DNA degradation obtained with Fe(II)·decarbamoyl-BLM A_2 was comparable to that obtained with Fe(II)·BLM A_2 (cf. lanes 2 and 3 with lanes 7 and 8, Figure 6), while that obtained with Fe(II)·deglyco-BLM was approximately half as great (cf. lanes 4–10, Figure 5), both in the presence and in the absence of dithiothreitol.

A comparison of DNA strand scission with these agents in the presence of Cu(II) + DTT was instructive. In parallel with Cu(II) + BLM + DTT, which effected strand scission about half as efficiently as Fe(II) + BLM + DTT (cf. lanes 10 and 16, Figure 5), the extent of cleavage obtained with Cu(II) + deglyco-BLM + DTT was a significant fraction of that obtained with Cu(II) + BLM + DTT (cf. lanes 13–16, Figure 5). In contrast, while DNA cleavage mediated by decarbamoyl-BLM A_2 + Fe(II) was quite similar to that obtained with BLM A_2 + Fe(II), DNA cleavage mediated in the presence of Cu(II) + decarbamoyl-BLM A_2 + DTT was much less efficient than that obtained with Cu(II) + BLM A_2 + DTT, even when the former was employed at much higher concentration (cf. lanes 4 and 9, Figure 6). In fact, DNA cleavage by Cu(II) + decarbamoyl-BLM A_2 + DTT was found to be less efficient than that mediated by Cu(II) + deglyco-BLM A_2 + DTT (cf. lanes 9–11, Figure 6, and lanes 14 and 15, Figure 5). Even more striking was the finding that admixture of Cu(II) + DTT + iso-BLM in the presence of DNA yielded no detectable DNA degradation, while Fe(II)·iso-BLM A_2 was as active as Fe(II)·BLM A_2 in DNA strand scission (Figure 7). In fact, Cu(II) + DTT + iso-BLM A_2 was found not to relax SV40 form I DNA (data not shown). If DNA cleavage mediated by Cu(II) in the presence of BLM A_2, deglyco-BLM A_2, and decarbamoyl-BLM A_2 were actually due to adventitious Fe, it would be difficult to see why the extent of DNA cleavage should not be in the same order as that observed for the authentic Fe derivatives of each BLM congener.

Additional qualitative evidence to distinguish between the activity of Cu·BLM and Fe·BLM was obtained by a study of the sequence specificity of DNA strand scission. As shown in Figure 8, for histograms of DNA cleavage patterns derived from Figure 6, relative cleavage efficiencies at certain sites differed significantly. More direct evidence of qualitative differences was obtained by analysis of the cleavage of a 127-base-pair 5′-^{32}P-end-labeled DNA duplex derived from SV40 DNA. As shown in Figure 9, significant differences in sequence specificity were noted when Fe·BLM A_2 and Cu·BLM A_2 were used to effect strand scission of this DNA duplex, a finding that strongly supports the existence of two

different activated metallobleomycins. Further supporting evidence was obtained from a study of the time course of activation of Fe·BLM and Cu·BLM. As shown in Figure 10, activation of Fe·BLM was complete within 15 s, as judged by the extent of DNA strand scission. On the other hand, Cu·BLM was not activated fully within the first 5 min, although the ultimate extent of DNA strand scission by both metallobleomycins (i.e., following full activation of each) was approximately the same. It is interesting to note that for Mn·BLM, which has also been noted to mediate DNA strand scission (Ehrenfeld et al., 1984; Burger et al., 1984; Suzuki et al., 1985a), measurement of the time course of activation indicated a profile not dissimilar to that noted for Cu·BLM (data not shown).

Oxygen Transfer to cis-Stilbene. We have demonstrated previously that activated BLM can effect oxidation of small substrates such as *cis*-stilbene in analogy with known monooxygenases and have shown that Cu·BLM and Fe·BLM gave oxidation products that differed both qualitatively and quantitatively following activation with iodosobenzene (Murugeson & Hecht, 1985). Presently, we have extended these findings by a more thorough quantitation of certain of the products formed (Table IV). As shown in the table, under the experimental conditions employed, the ratio of *trans*-stilbene oxide:*cis*-stilbene oxide formed was greater when Cu(II)·BLM + C_6H_5IO was used (0.25 vs. 0.038 at 1:1 metal ion:BLM), while deoxybenzoin was formed only with Fe(III)·BLM + C_6H_5IO. To explore the possibility that metallobleomycin-mediated oxidations may employ more than one metal atom per BLM molecule, was also studied the oxidation of *cis*-stilbene in the presence of BLM and excess metal ion. As shown in Table IV, a dramatic increase in product yield was noted when a 5-fold excess of Cu(II) was employed. In the case of Fe(III), the addition of excess metal actually diminished the observed overall yields of products. Although the molecular basis for the results in this table is not altogether clear at present, they provide strong support for the concept of different active metallobleomycins formed from Cu·BLM and Fe·BLM.

Conclusions

Consistent with earlier work, the present study demonstrates Cu-dependent, bleomycin-mediated DNA strand scission and olefin oxygenation. Activation of bleomycin in the presence of Cu was shown to depend importantly on the order of addition of reagents and to proceed over a different time course than that of Fe·BLM. Qualitative differences observed between Cu·BLM and Fe·BLM included the pattern of DNA strand scission obtained with a 127-base-pair restriction fragment, as well as the type and amount of products formed during oxygenation of *cis*-stilbene.

Acknowledgments

We thank Drs. L. H. Chang and L. T. Ma for samples of the modified BLM's employed in this study.

Supplementary Material Available

Figures 1 and 2 showing respectively the effect of DTT on sequence selectivity of DNA cleavage by Fe(II)·BLM and variations in band intensity during replicate digestions of ^{32}P-end-labeled DNA with Fe(II)·BLM (2 pages). Ordering information is given on any current masthead page.

Registry No. Deglyco-BLM A_2, 78193-35-8; decarbamoyl-BLM A_2, 106064-23-7; iso-BLM A_2, 51041-93-1; BLM B_2, 9060-10-0; BLM A_2, 11116-31-7; Cu(II)-BLM A_2, 71794-63-3; Cu(I)-BLM A_2, 72348-90-4; Fe(II)-BLM A_2, 71801-37-1; Cu, 7440-50-8; Fe, 7439-89-6; PhCHO, 100-52-7; $PhCH_2COPh$, 451-40-1; d-(CGCTTTAAAGCG), 97920-47-3; *cis*-stilbene, 645-49-8; *trans*-stilbene oxide, 1439-07-2; *cis*-stilbene oxide, 1689-71-0; deferoxamine mesylate, 138-14-7; Cu(II) deferoxamine mesylate, 106064-25-9; Cu(I) deferoxamine mesylate, 106064-27-1; Cu(II) bathocuproine mesylate, 106064-28-2; Cu(I) bathocuproinedisulfonate, 106064-29-3; bathocuproinedisulfonate, 73348-75-1.

References

Aft, R. L., & Mueller, G. C. (1983) *J. Biol. Chem. 258*, 12069–12072.

Albertini, J.-P., & Garnier-Suillerot, A. (1982) *Biochemistry 21*, 6777–6782.

Antholine, W. E., Solaiman, D., Saryan, L. A., & Petering, D. H. (1982) *J. Inorg. Biochem. 17*, 75–94.

Antholine, W. E., Hyde, J. S., Sealy, R. C., & Petering, D. H. (1984) *J. Biol. Chem. 259*, 4437–4440.

Aoyagi, Y., Suguna, H., Murugesan, N., Ehrenfeld, G. M., Chang, L.-H., Ohgi, T., Shekhani, M. S., Kirkup, M. P., & Hecht, S. M. (1982) *J. Am. Chem. Soc. 104*, 5237–5239.

Bereman, R. D., & Winkler, M. E. (1980) *J. Inorg. Biochem. 13*, 95–104.

Burger, R. M., Freedman, J. H., Horwitz, S. B., & Peisach, J. (1984) *Inorg. Chem. 23*, 2217–2219.

Chang, C.-H., & Meares, C. F. (1982) *Biochemistry 21*, 6332–6334.

Chang, C.-H., & Meares, C. F. (1984) *Biochemistry 23*, 2268–2274.

Chien, M., Grollman, A. P., & Horwitz, S. B. (1977) *Biochemistry 16*, 3641–3646.

Chu, B. C. F., & Orgel, L. E. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 963–967.

Cotton, F. A., & Wilkinson, G. (1972) in *Advanced Inorganic Chemistry*, pp 905–911, Wiley-Interscience, New York.

Crooke, S. T., & Bradner, W. T. (1977) *J. Med. (Westbury, N.Y.) 7*, 333–428.

Dabrowiak, J. C. (1980) *J. Inorg. Biochem. 13*, 317–337.

D'Andrea, A. D., & Haseltine, W. A. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3608–3612.

Downey, K. M., Que, B. G., & So, A. G. (1980) *Biochem. Biophys. Res. Commun. 93*, 264–270.

Dreyer, G. B., & Dervan, P. B. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 968–972.

Ehrenfeld, G. M., Murugesan, N., & Hecht, S. M. (1984) *Inorg. Chem. 23*, 1498–1500.

Ehrenfeld, G. M., Rodriquez, L. O., Hecht, S. M., Chang, C., Basus, V. J., & Oppenheimer, N. J. (1985) *Biochemistry 24*, 81–92.

Eliot, H., Gianni, L., & Myers, C. (1984) *Biochemistry 23*, 928–936.

Emery, T. (1971) *Adv. Enzymol. Relat. Areas Mol. Biol. 35*, 135–183.

Fiel, R. J., Beerman, T. A., Mark, E. H., & Datta-Gupta, N. (1982) *Biochem. Biophys. Res. Commun. 107*, 1067–1074.

Freedman, J. H., Horwitz, S. B., & Peisach, J. (1982) *Biochemistry 21*, 2203–2210.

Goldstein, S., & Czapski, G. (1986) *J. Am. Chem. Soc. 108*, 2244–2250.

Graham, D. R., Marshall, L. E., Reich, L. E., & Sigman, D. S. (1980) *J. Am. Chem. Soc. 102*, 5421–5423.

Halliwell, B. (1985) *Biochem. Pharmacol. 34*, 229–233.

Hashimoto, Y., Iijima, H., & Shudo, K. (1984) *Gann 75*, 567–570.

Hecht, S. M., Ed. (1979) *Bleomycin: Chemical, Biochemical and Biological Aspects*, Springer-Verlag, New York.

Hênichart, J.-P., Houssin, R., Bernier, J.-L., & Catteau, J.-P. (1982) *J. Chem. Soc., Chem. Commun.*, 1295–1297.

Hertzberg, R. P., & Dervan, P. B. (1984) *Biochemistry 23*, 3934–3945.
Hoe, S., Rowley, D. A., & Halliwell, B. (1982) *Chem.-Biol. Interact. 41*, 75–81.
Iitaka, Y., Nakamura, H., Nakatani, T., Muraoka, Y., Fujii, A., Takita, T., & Umezawa, H. (1978) *J. Antibiot. 31*, 1070–1072.
Ishida, R., & Takahashi, T. (1975) *Biochem. Biophys. Res. Commun. 66*, 1432–1438.
Ishizuka, M., Takayama, H., Takeuchi, T., & Umezawa, H. (1967) *J. Antibiot., Ser. A 20*, 15–24.
Jessee, B., Gargiulo, G. Razvi, F., & Worcel, A. (1982) *Nucleic Acids Res. 10*, 5823–5834.
Kano, M., Tomita, S., Ishida, S., Murakami, A., & Okada, H. (1973) *Chemotherapy (Tokyo) 21*, 1305–1310.
Kazmi, S. A., & McArdle, J. V. (1981) *J. Inorg. Biochem. 15*, 153–162.
Kilkuskie, R. E., Macdonald, T. L., & Hecht, S. M. (1984) *Biochemistry 23*, 6165–6171.
Kross, J., Henner, W. D., Hecht, S. M., & Haseltine, W. A. (1982) *Biochemistry 21*, 4310–4318.
Kuwahara, J., Suzuki, T., & Sugiura, Y. (1985) *Biochem. Biophys. Res. Commun. 129*, 368–374.
Lown, J. W., & Joshua, A. V. (1982) *J. Chem. Soc., Chem. Commun.*, 1298–1300.
Maniatis, T., Fritsch, E., & Sambrook, J. (1982) *Molecular Cloning*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
Marshall, L. E., Graham, D. R., Reich, K. A., & Sigman, D. S. (1981) *Biochemistry 20*, 244–250.
Maxam, A. M., & Gilbert, W. (1980) *Methods Enzymol. 65*, 499–560.
Mirabelli, C. K., Huang, C.-H., & Crooke, S. T. (1983) *Biochemistry 22*, 300–306.
Muraoka, Y., Suzuki, M., Fujii, A., Umezawa, Y., Naganawa, H., Takita, T., & Umezawa, H. (1981) *J. Antibiot. 34*, 353–357.
Murugesan, N., & Hecht, S. M. (1985) *J. Am. Chem. Soc. 107*, 493–500.
Murugesan, N., Ehrenfeld, G. M., & Hecht, S. M. (1982) *J. Biol. Chem. 257*, 8600–8603.
Murugesan, N., Xu, C., Ehrenfeld, G. M., Sugiyama, H., Kilkuskie, R. E., Rodriguez, L. O., Chang, L.-H., & Hecht, S. M. (1985) *Biochemistry 24*, 5735–5744.
Naganawa, H., Muraoka, Y., Takita, T., & Umezawa, H. (1977) *J. Antibiot. 30*, 388–396.
Nakayama, Y., Kurushima, M., Omoto, S., Takita, T., & Umezawa, H. (1973) *J. Antibiot. 26*, 400–402.
Nunn, A. D., & Lunec, J. (1978) *Eur. J. Cancer 14*, 857–863.
Oppenheimer, N. J., Rodriquez, L. O., & Hecht, S. M. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 5616–5620.
Oppenheimer, N. J., Chang, C., Rodriguez, L. O., & Hecht, S. M. (1981) *J. Biol. Chem. 256*, 1514–1517.
Oppenheimer, N. J., Chang, C., Chang, L.-H., Ehrenfeld, G., Rodriguez, L. O., & Hecht, S. M. (1982) *J. Biol. Chem. 257*, 1606–1609.
Pope, L. M., Reich, K. A., Graham, D. R., & Sigman, D. S. (1982) *J. Biol. Chem. 257*, 12121–12128.
Povirk, L. F., Hogan, M., Dattagupta, N., & Buechner, M. (1981) *Biochemistry 20*, 665–671.
Que, B. G., Downey, K. M., & So, A. G. (1980) *Biochemistry 19*, 5987–5991.
Rao, E. A., Saryan, L. A., Antholine, W. E., & Petering, D. H. (1980) *J. Med. Chem. 23*, 1310–1318.
Reich, K. A., Marshall, L. E., Graham, D. R., & Sigman, D. S. (1981) *J. Am. Chem. Soc. 103*, 3582–3584.
Sanchez-Rasero, F. (1981) *Microchem. J. 26*, 418–425.
Sanger, F., & Coulson, A. R. (1975) *J. Mol. Biol. 94*, 441–448.
Sausville, E. A., Peisach, J., & Horwitz, S. B. (1978a) *Biochemistry 17*, 2740–2746.
Sausville, E. A., Stein, R. W., Peisach, J., & Horwitz, S. B. (1978b) *Biochemistry 17*, 2746–2754.
Shirakawa, I., Azegami, M., Sin-ichi, I., & Umezawa, H. (1971) *J. Antibiot. 24*, 761–766.
Sigman, D. S., Graham, D. R., D'Aurora, V., & Stern, A. M. (1979) *J. Biol. Chem. 254*, 12269–12272.
Smith, G. F., & Wilkins, D. H. (1953) *Anal. Chem. 25*, 510–511.
Solaiman, D., Rao, E. A., Antholine, W., & Petering, D. H. (1980) *J. Inorg. Biochem. 12*, 201–220.
Sugiura, Y. (1979) *Biochem. Biophys. Res. Commun. 90*, 375–383.
Sugiura, Y. (1980) *J. Am. Chem. Soc. 102*, 5216–5221.
Sugiura, Y., Ishizu, K., & Miyoshi, K. (1979) *J. Antibiot. 32*, 453–461.
Sugiyama, H., Ehrenfeld, G. M., Shipley, J. B., Kilkuskie, R. E., Chang, L.-H., & Hecht, S. M. (1985a) *J. Nat. Prod. 48*, 869–877.
Sugiyama, H., Kilkuskie, R. E., Hecht, S. M., van der Marel, G. A., & van Boom, J. H. (1985b) *J. Am. Chem. Soc. 107*, 7765–7767.
Sugiyama, H., Kilkuskie, R. E., Chang, L.-H., Ma, L.-T., Hecht, S. M., van der Marel, G. A., & van Boom, J. H. (1986) *J. Am. Chem. Soc. 108*, 3852–3854.
Suzuki, H., Kirino, Y., & Tanaka, N. (1983) *J. Antibiot. 36*, 583–587.
Suzuki, T., Kuwahara, J., Goto, M., & Sugiura, Y. (1985a) *Biochim. Biophys. Acta 824*, 330–335.
Suzuki, T., Kuwahara, J., & Sugiura, Y. (1985b) *Biochemistry 24*, 4719–4721.
Takahashi, K., Yoshioka, O., Matsuda, A., & Umezawa, H. (1977) *J. Antibiot. 30*, 861–869.
Takeshita, M., Grollman, A. P., Ohtsubo, E., & Ohtsubo, H. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5983–5987.
Takita, T., Muraoka, Y., Nakatani, T., Fujii, A., Iitaka, Y., & Umezawa, H. (1978) *J. Antibiot. 31*, 1073–1077.
Taylor, J. S., Schultz, P. G., & Dervan, P. B. (1984) *Tetrahedron 40*, 457–466.
Uesugi, S., Shida, T., Ikehara, M., Kobayashi, Y., & Kyogoku, Y. (1982) *J. Am. Chem. Soc. 104*, 5494–5495.
Umezawa, H., Masaaki, I., Kimura, K., Iwanaga, J., & Takeuchi, T. (1968) *J. Antibiot. 21*, 592–602.
Umezawa, H., Asakura, H., Oda, K., & Hori, S. (1973) *J. Antibiot. 26*, 521–527.
Wong, A., Huang, C.-H., & Crooke, S. T. (1984a) *Biochemistry 23*, 2939–2945.
Wong, A., Huang, C.-H., & Crooke, S. T. (1984b) *Biochemistry 23*, 2946–2952.