Drug/Nucleic Acid Interactions: Double Strand Cleavage of DNA by Bleomycin

by

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DEDICATION

My family has been kind to me. I owe them a great debt. My parents and mother-in-law helped us keep body and soul together. My wife and children shared with me the difficulties and the joys of this endeavor. I dedicate this work and its completion to them.

PREFACE

We have made use of many techniques developed in the field of molecular biology. The adaptation of these methods to the study of drug/nucleic acid interactions has allowed new questions to be asked, and answered, for the important class of drugs with nucleic acids as targets. In return, it is hoped, these studies will provide new information of interest to the biochemist. For example, the design, synthesis, and study of novel DNA sequences of interest to the medicinal chemist, can yield new insights about DNA structure and dynamics. Likewise, the use of unusual DNA sequences, designed to serve as models for drug/DNA interactions, has led to improvements in the techniques of analytical biochemistry.

The Department of Pharmaceutical Chemistry at U.C.S.F. is a remarkable research center. I am deeply appreciative of the opportunity to study and work in what has always been a stimulating and intellectually supportive environment. The entire faculty is outstanding, but in particular I would like to acknowledge and thank my research advisor, Dr. Norman Oppenheimer, and Drs. Dick Shafer, C.C. Wang, Tack Kuntz, Frank Szoka, Alice Wang, Judith McClarin and Peter Kollman for fruitful discussions and advice. Above all, I wish to thank them for serving as models of the highest quality scholarship.

My research took me outside of the expertise of my department. I would like to acknowledge and thank Paula Szoka, of the Syntex Corporation, for introducing me to the techniques of molecular biology; and Fran DeNoto, of U.C.S.F., for sharing her wealth of practical knowledge in this field. The technical assistance

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ABSTRACT: The interaction of the anitcancer drug, bleomycin, with its target DNA has been investigated in the hope of answering several basic questions pertinent to the drug's mechanism of action. Our initial interest was to determine whether bleomycin showed any extended sequence specificity in its generation of double strand breaks. That is, does bleomycin "recognize", e.g. by means of specific hydrogen bonds or salt bridges, a stretch of the DNA sequence at those sites where rapid, double strand breakage occurs. The techniques used to study the specificity of restriction enzymes were adapted for this purpose. No extended recognition sequence could be identified. However, the pursuit of this question led to a more detailed investigation of the mechanism of double strand breaks than had heretofore been accomplished. To this end, we studied the cleavage, by bleomycin, of several synthetic oligonucleotides of specified sequences. In addition, oligonucleotides were modified by chemical and enzymatic means to construct duplex models of bleomycin nicked DNA. A bleomycin generated nick on one strand leaves a gap of one nucleotide bounded by dianionic phosphate moieties. Experiments using the oligonucleotide nick models demonstrate that the increased negative charge bounding the gap directs cleavage by the cationic bleomycin to the opposite strand. This mechanism can explain the finding that bleomycin generates double strand breaks more rapidly than expected by the random accumulation of single strand breaks.

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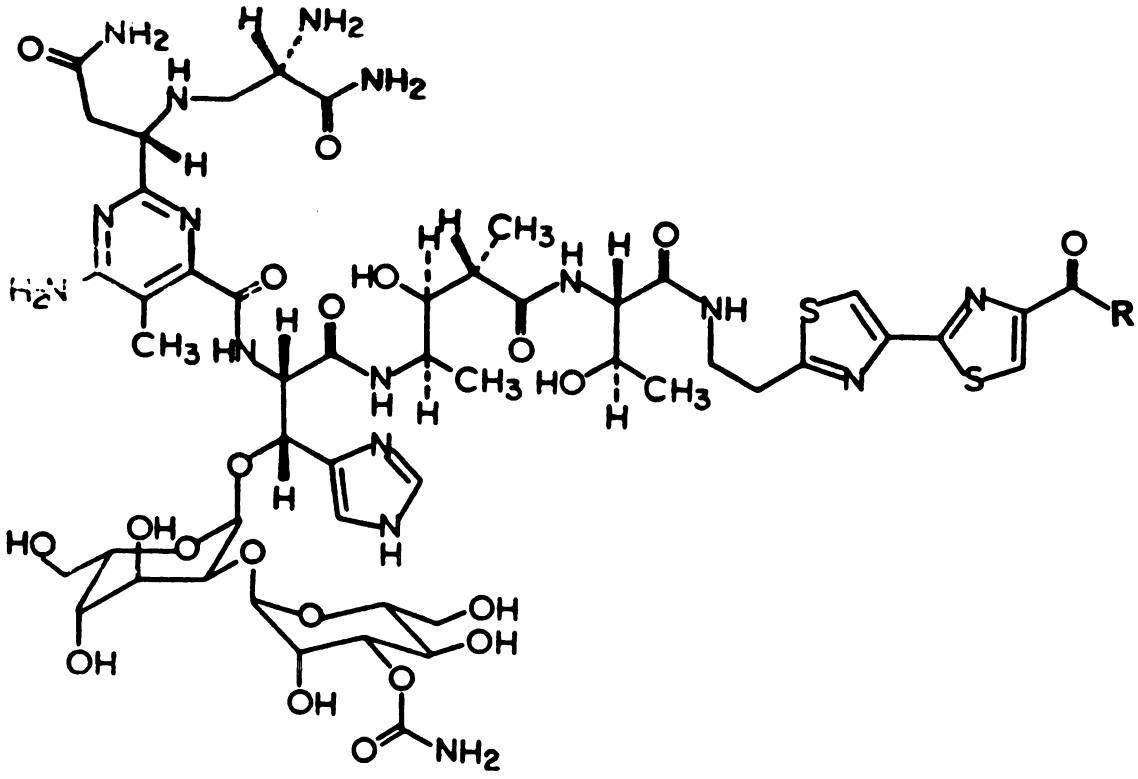
I. BACKGROUND

The Bleomycins are a family of glycopeptide antibiotics isolated as the copper chelates from Streptomyces verticillus. They were discovered in the early 1960's by a Japanese research group headed by Dr. Hamao Umezawa. The clinically used mixture, Blenoxane, is approximately 55-70% bleomycin A2 and 25-30% bleomycin B2, with several minor components. Their clinical usefulness against certain kinds of tumors, especially squamous cell carcimomas, Hodgekin's lymphoma, and testicular carcinoma, was established by 1966. Of interest in this regard is bleomycin's relative lack of immune system suppression and damage to bone marrow (Umezawa et al., 1966; Umezawa et al., 1979;). This characteristic of the drug's distribution and metabolism allows an enhancement of chemotherapy by combination of bleomycin with other antineoplastic agents. Additionally, bleomycin has been used as adjuvant therapy after surgical treatment of cancer (Bitran et al., 1981). The dose limiting toxic effect is pulmonary fibrosis. The degree of this effect is different for the various bleomycin types (see *figure* 1) (Carter et al., 1976).

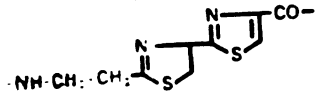
***Figure* 1**

Structure of Metal Free Bleomycins and Congeners



Modifications constituting congeners:

phleomycin



tallysomycin

L-threonine

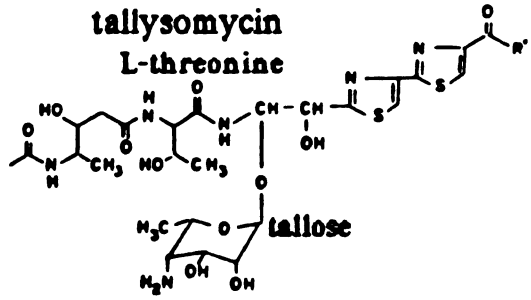
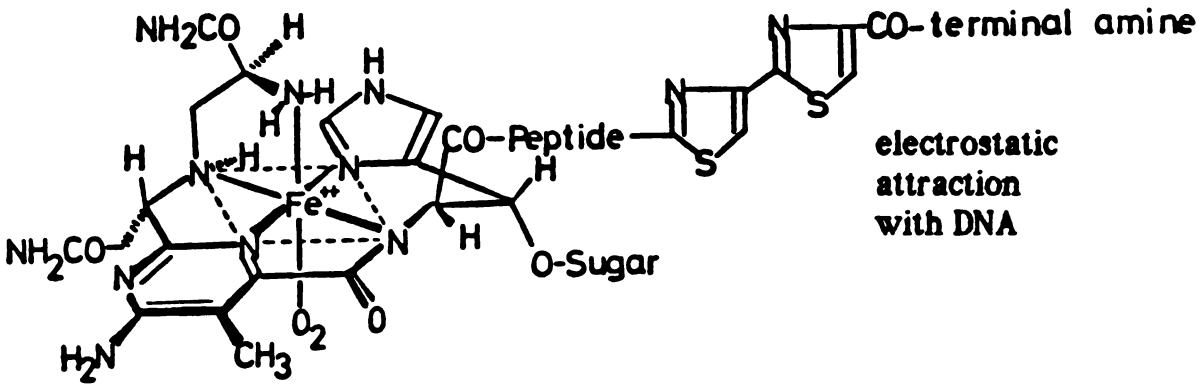


Figure 2

Metal-bleomycin Complex



Metal binding domain

DNA binding domain

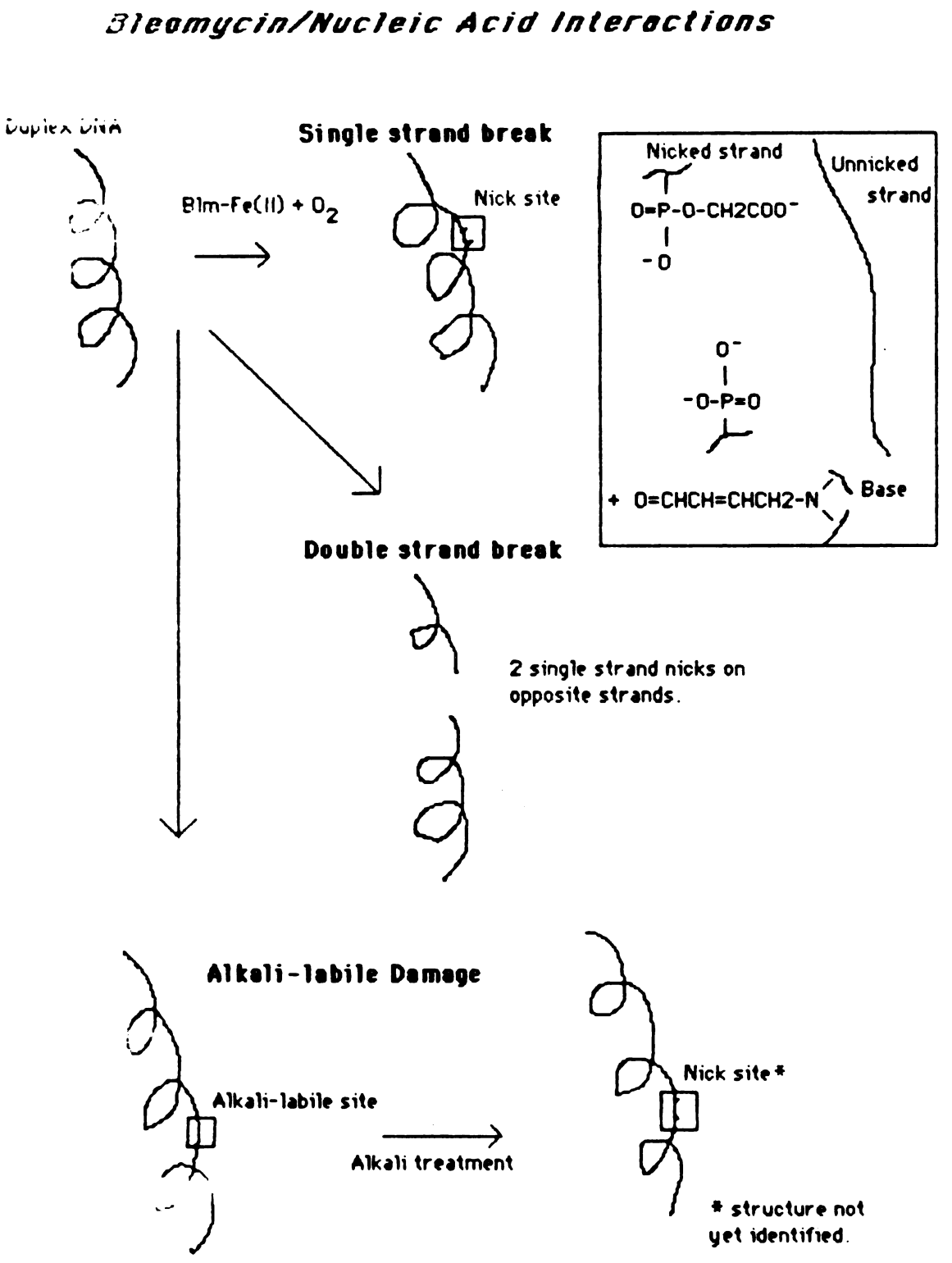
Biochemical mechanism of action of the drug.

Bleomycin inhibits cell proliferation in both prokaryotes and eukaryotes (Müller et al., 1977). It strongly affects DNA synthesis, while RNA and protein synthesis are much less effected (Müller et al.,1975). The diminuation of DNA synthesis was traced to inhibition of DNA dependent polymerases due to modification of the DNA substrate (Yamazaki et al.,1973; DiCioccio et al., 1976; Müller et al., 1973). Bleomycin causes fragmentation of viral, bacterial, and mammalian cell DNA (Takeshita et al., 1974;Onishi et al., 1973; Müller et al., 1973).9 *In vitro* studies of leukemia cells found cytotoxicity to correlate with the amount of strand breakage produced by the drug, e.g. those factors that enhanced *in vitro* DNA strand cleavage also promoted the drug's *in vivo* cell killing ability. Müller and co-workers (1973) showed that excess bleomycin, *in vivo*, gave a drastic reduction of deoxythymidine incorporation, yet uridine and tryptophan incorporation were not significantly reduced. Umezawa and co-workers also demonstrated unbalanced growth of cells, with inhibition of DNA synthesis while protein and RNA synthesis continued (Suzuki et al., 1968). Finally, bleomycin cleaves DNA within the nucleus of cells, causing release of nucleosomal DNA (Kuo,1981).

Bleomycin generates oxidative cleavage of the DNA backbone (Suzuki et al., 1969; Haidle, 1971). The chemistry of the reaction will be discussed below. The nicks can occur as isolated single strand breaks or within a short distance of each other on opposite strands to form double strand breaks. In addition, bleomycin can generate alkali-labile sites with release of the free bases (see *figure* 3).

While all three types of bleomycin generated damage are repaired, double strand breaks are repaired much more slowly than single strand breaks (Saito et al., 1973). In addition, recent research has demonstrated that for a given level of cell growth inhibition, different bleomycin congeners cause quite different amounts of DNA cleavage (Berry et al., 1985). It appears that the net amount of DNA cleavage by bleomycin may not be the major determinant of its antineoplastic activity. Several alternatives are possible, including the location of the damage, the amount of double strand cleavage rather than single strand breakage, or the refractoriness of particular damage to repair synthesis.

Figure 3


Bleomycin/Nucleic Acid Interactions
Duplex DNA
Single strand break
Blm-Fe(II) + O_2
Nick site
Nicked strand
Unnicked strand
O=P-O-CH2COO⁻
-O
O⁻
-O-P=O
+ O=CHCH=CHCH2-N
Base
Double strand break
2 single strand nicks on opposite strands.
Alkali-labile Damage
Alkali-labile site
Alkali treatment
Nick site*
* structure not yet identified.

Structure

The complete structure of the bleomycins was more difficult to establish than its clinical usefulness. Accurate mass data has proved illusive; neither mass spectrometry (including field desorption) nor crystallization has been achieved on the entire molecule. Therefore, wet chemical analysis and 13C NMR were the principle tools used to determine the structure originally proposed by T. Takita and co-workers (1972). Subsequent crystallization and x-ray analysis of the copper complex of a peptide precursor of bleomycin and further NMR work led to the revised structure of the parent compound, bleomycinic acid, shown in *figure* 1 (Takita et al., 1978). Several of the terminal amine moieties, which differentiate the various bleomycins, are also shown. The molecular weight of the major component of the clinical mixture, bleomycin A2, is 1423 daltons. The extinction coeficient is 14,100 at 287 nm (Dabrowiak et al., 1979). Bleomycin has a high affinity for divalent transition metal ions (Takita et al., 1978). The structures of various metal-bleomycin complexes have been studied (Oppenheimer et al., 1979 and 1980). It is known that analogues of bleomycin with differing geometries around the metal are capable of degrading DNA, the target of bleomycin (Oppenheimer et al., 1982) *Figure* 2, shows a model of the metal complex consistent with most of the data to date.

II. BLEOMYCIN/NUCLEIC ACID INTERACTIONS

Reaction with DNA

Investigation of the chemistry of the cleavage reaction has been approached in several ways. The drug's reaction with thymidine labelled poly (dA-dT) showed the capacity of the drug to cause release from DNA of free (unsubstituted) bases

as well as bases containing an aldehyde moiety (Müller et al., 1972; Haidle et al., 1972). The modified base, produced by the DNA cleavage, reacted with thiobarbituric acid and as such was identified as malondialdehyde (Gutteridge, 1979).1 Subsequent research demonstrated that the base is released attached to a 2–propenal fragment of the deoxyribose ring (Giloni et al., 1981). This yields malondialdehyde upon reaction with thiobarbituric acid. Burger and co-workers isolated three sequentially related products from bleomycin damaged DNA, the last one indistinguishable from authentic malondialdehyde (Burger et al., 1980).

Grollman and co-workers (1981) also demonstrated that the 4' and 5' carbons of the attacked deoxyribose are released from the DNA strand as a glycolic acid moiety. The glycolate group was confirmed as being directly attached to the remaining 3' terminal phosphate after bleomycin cleavage, see *figure* 3 (Uesugi et al., 1984).

Early work on the chemistry of this oxidative reaction correlated the quenching of the bleomycin fluorescence by ferrous ion with DNA breakage activity (Huang et al., 1979). The degradation of DNA by bleomycin requires Fe(II), Fe(III) was not effective. Removal of oxygen inhibits the reaction and optical spectra indicated an oxygen-labile complex is formed by bleomycin and Fe(II) (Sauseville et al.,1977). Subsequent research has demonstrated other redox active metals to effectively substitute for Fe(II), e.g. Cu(I) and Co(III) Deremier et al., 1979; Oppenheimer et al., 1981).

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[1] This researcher noted that malondialdehyde is reactive and could cause secondary cross-linking damage. The chemistry of malondialdehyde in biological systems has been studied (Nair et al., 1981).

The release of bases from DNA was shown to be catalytic (Povirk,1979). The oxygen-iron-bleomycin complex cycles between +2 and +3 oxidation states to catalyze the generation of an activated oxygen species, with thiols able to serve as a source of electrons (Antholine et al., 1979). The redox potential, 129 +/- 12 mV (25°, pH 7.0), of the bleomycin-Fe(III)/Fe(II) couple is in the range of several cellular reductants; among them NADH (–318mV), ascorbate (-58mV), and glutathione (-230mV) (Melynk et al., 1981). The kinetics of the reaction of Fe-bleomycin with oxygen has been studied by Burger and co-workers (1979). The time course of the changes of the Fe-bleomycin epr signal are comparable to that of the base release activity (Kuramochi et al., 1981).

Grollman and co-workers proposed the cleavage reaction to occur via radical abstraction of the hydrogen at the deoxyribose 4' position by a bleomycin generated, activated oxygen species. Under aerobic conditions, this could be followed by formation of an unstable peroxide species. The collapse of this species, with cleavage of the deoxyribose C3-C4 bond, would form the identified products (Giloni et al., 1981). A diagram showing this reaction is shown in *figure* 4.

Corroborative evidence for this mechanism has been obtained. Wu and co-workers tracked the distribution of radioisotopic labels at the 1', 2', 3', and 4' postion of deoxyribose of poly(dA-dU) in the products of the bleomycin reaction (Wu et al., 1983). Their results are consistent with the formation of unsubstituted base and base-propenal by subsequent reaction after abstraction of the 4' hydrogen by a bleomycin generated activated oxygen species.

The distribution of products is sensitive to the reaction conditions. Reductants such as dithiothreitol or beta-mercaptoethanol promote the cleavage reaction, presumably by maintaining the reduced iron concentration (Ross and Moses, 1978). Partitioning between unsubstituted base release vs base-propenal generation was demonstrated to be a function of the oxygen concentration, O2 promotes strand cleavage (Burger et al., 1982). Kozarich and co-workers proposed a single intermediate for the two types of damage, see *figure* 4. However, the exact mechanism differentiating release of unsubstituted base, leaving an intact but alkali-labile strand, from strand cleavage with the generation of a base-propenal is not completely understood (Wu et al., 1983).

The hydrogen abstraction chemistry of bleomycin is reminsicent of the first step of carbon hydroxylation by P450 and its redox chemistry with iron and molecular oxygen (Guengerich et al., 1984). Like cytochrome P-450, reduced Fe(III)-bleomycin and several of its structural analogues can use oxidants such as iodosobenzene or periodate to form an active complex cabable of degrading DNA. The activated complex can also transfer oxygen stereoselectively to various olefinic substrates, analogous to P450 (Murugesan and Hecht, 1985).

Figure 4
Reaction with DNA; current model

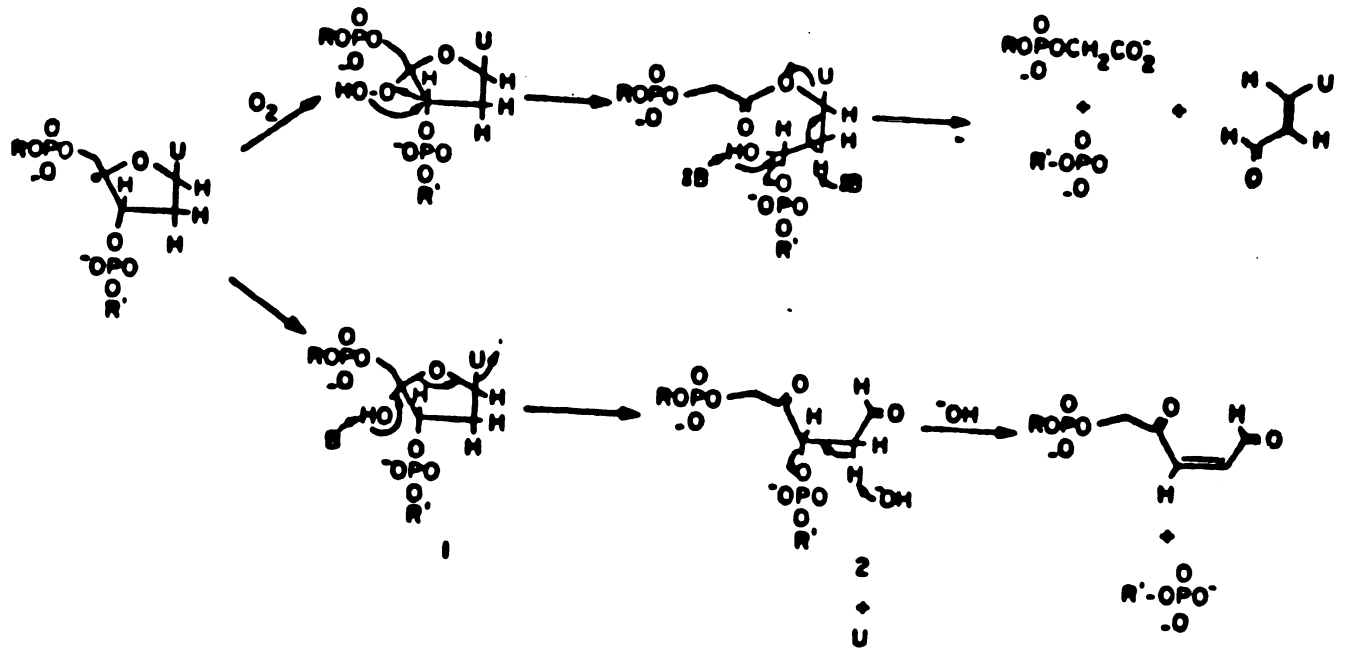

Figure 4

1. Grollman et al., (1981)
2. Wu, Kozarich, & Stubbe (1983)

Binding to DNA

Investigation of the chemistry and biochemistry of bleomycin's antineoplastic activity has focused on the drug's interaction with nucleic acids. Chien and co-workers (1977), used the fluorescence quenching of the bithiazole moiety of bleomycin when bound to DNA to determine the equilibrium binding constant, (Keq), and the number of drug molecules bound per nucleotide, (n), for metal free bleomycin with calf thymus DNA. In 2.5 mM Tris (pH 8.4), Keq = 1.2 X 10-5 M, this drops by about 1/4 with the addition of 50 mM MgCl2. This suggests electrostatic interactions play a significant role in DNA binding. Approximately one bleomycin was bound per 5 or 6 nucleotides. Metal-bleomycin complexes can bind to DNA significantly better than metal-free bleomycin. For example, Co(III)-bleomycin and Cu(I)-bleomycin have binding constants on the order of 10-7 M (Chang and Meares, 1984).

More detailed studies of the binding of the drug to its target allow several facets of the interaction to be differentiated. Bleomycin shows definite preferences for the polynucleotide structure, primary, secondary, and tertiary. The most complete study of bleomycin's binding behaviour was conducted by M. Hori and co-workers (1979). In these experiments, the cleavage reaction of bleomycin with SV 40 DNA (RF I) was monitored as a function of competing nucleic acids of various sorts. Neither RNA nor single stranded polynucleotides could compete for bleomycin binding with the duplex DNA. Mixed sequence polynucleotides (duplex) containing deoxyguanosine offered more protection of the test DNA than other types of polynucleotides. An alternative experimental approach, monitoring the fluorescence quenching of the bleomycin bithiazole moiety upon addition of various polynucleotides and nucleotides did not confirm this preference of bleomycin for duplex DNA (Kasai et al., 1978). We conclude

that an interaction sufficient to cause quenching of the bithiazole fluorescence occurs with most polynucleotides, and especially those containing guanosine. However, bleomycin shows a strong preference for duplex DNA. We have demonstrated that bleomycin can cleave duplex DNA as small as a hexamer, but the isolated, single strands of the hexamer are not cleaved by the drug (vide infra). In short, bleomycin binds preferentially to duplex, poly and oligodeoxynucleotides of mixed sequence that includes dG.

The concept of two functional domains suggests itself from the structural studies of bleomycin. These suggest a metal binding portion and a DNA binding portion. The latter consists of a short intervening peptide region and the bithiazole rings with a cationic amine substituent (see *figure* 2). Early studies of metal free bleomycin demonstrated the bithiazole and terminal amine were most perturbed by addition of DNA. Chien and co-workers (1977), using NMR, found the proton resonances of bithiazole and the dimethylsulfonium groups were preferentially broadened in the presence of DNA; they suggested these groups were bound most tightly to the DNA.

The metal-bleomycin complex may behave somewhat differently. Antholine and co-workers (1981) compared the perturbations of the proton resonances of the carbon monoxide complex of iron(II)-bleomycin with metal free bleomycin in the presence of poly(dA-dT). They confirmed the shifting and broadening of the bithiazole resonaces as the major effect of metal free bleomycin. In addition, they found that the two imidazole resonances and the methylpyrimidine resonance of CO-Fe(II)-bleomycin were also effected by binding to DNA (Antholine et al., 1981).

The independence of the metal binding region of the molecule was tested by the model studies of Umezawa and co-workers (1984). They synthesized a minimal metal binding site capable of activating oxygen (see *figure* 1). As depicted in *figure* 2, the pyrimidine and imidazole groups were required to be trans. Kilkuskie and co-workers (1985) also studied the cleavage of DNA by bleomycin analogues lacking the putative DNA binding domain. As in the previous experiment, the analogues can bind and activate oxygen, analagous to iron-bleomycin (vide supra). However, they can only randomly nick DNA to the same extent as Fe(II) alone, i.e. like Fenton's reagent.

Several groups have synthesized analogues of the bithiazole moiety as models of the putative DNA binding portion of the molecule (Sakai et al., 1983; Kross et al., 1982). These compounds lack the metal binding domain and are completely inactive at cleaving DNA. Yet the physical properties of their interaction with DNA, e.g. binding constants and hydrodynamic properties, are qualitatively similar to those of the corresponding groups of intact bleomycin. The conclusion of this work was that the dominant features for optimal binding of bleomycin are the presence of at least two thiazole rings, and the importance of the length of the cationic side chain. These findings are generally consistent with *figure* 2.

The actual mode of binding to DNA by bleomycin is still under investigation. Intercalation of the coplanar bithiazole rings has been suggested Koyama et al., 1968). Several findings support this hypothesis. The NMR indicates significant interaction of the bithiazole rings and the cationic terminal moiety of bleomycin with DNA (Chien et al., 1977). Povirk and co-workers studied the hydrodynamic properties of DNA in the presence of bleomycin and Tripeptide S, the bleomycin hydrolysis fragment consisting only of methylvalerate, bithiazole, and the

terminal amine. Metal free bleomycin lengthens linear DNA to the same extent as Tripeptide S. Tripeptide S both unwinds and rewinds supercoiled DNA. However, metal free bleomycin was able only to unwind the supercoiled DNA, it did not induce rewinding of the supercoils as expected for an intercalator (Povirk et al., 1979). These researchers suggested that, even at the low pH used to inactivate the drug, the higher concentrations needed to produce supercoiling in the opposite sense caused nicking, and hence relaxation of the circular DNA. Finally, several other analogues of the bithiazole and terminal amine portion of bleomycin have been used as probes for the mode of binding of the drug (Fisher et al.,1985). DNA was incubated with bleomycin in the presence of the model compounds and the amount of DNA cleavage was assayed. The analogues that were likely intercalators were also the best inhibitors of bleomycin cleavage activity.

The evidence for intercalation is, however, far from complete. The NMR results, for example, show a curious temperature dependence. The greatest shift of the bithiazole proton resonances occurred at 50°C, just prior to melting of the duplex structure. At higher temperatures the shifts were lost, and at lower temperatures the shifts were significantly reduced (Chen et al., 1980; Sakai et al., 1983). This suggests that a significant amount of unwinding of the DNA helical structure must occur before the bithiazole rings are able to fully intercalate. Partial intercalation may occur. However, full intercalation of the bithiazole rings does not appear to be the dominant factor for DNA binding under normal conditions.

The significance of electrostatic interactions between bleomycin and DNA is well established. As mentioned above, the binding constant is very sensitive to

ionic strength (Chien et al.,1977). Analogues modeling the putative DNA binding portion of bleomycin demonstrate a substantial contribution of the cationic side chain to the binding energy (Sakai et al., (1983); Kross et al., 1982). Finally, the ratio of double strand breaks to single strand breaks is also sensitive to ionic strength. Lloyd and co-workers (1978) found the initial rate of double strand breaks was more drastically reduced than the rate of single strand breaks by increasing ionic strength. This suggests the significance of electrostatic interactions between bleomycin and DNA for the generation of double strand breaks as well as single strand breaks.

Specificity of single strand cleavage.

Interest in the interaction of bleomycin with DNA was greatly stimulated when it was discovered that pyrimidines are preferentially released from the reaction (Povirk et al.,1978). Although high concentrations of the drug cause extensive damage to DNA, the cleavage reaction does not occur randomly.2 The specificity shown by bleomycin in producing both single strand breaks and double strand breaks has been a major focus of research.

Understanding the specificity of bleomycin's generation of single strand breaks is required for an understanding of double strand cleavage by the drug. The technique of chemical sequencing of DNA (Maxam and Gilbert, 1980) has been successfully applied to DNA cleaving drugs, including bleomycin. The sequence specificity of bleomycin generated single strand breaks has been determined by several groups. The preference of bleomycin for (5'—>3') GC and GT sequences was demonstrated by comparing the fragments produced by a bleomycin reaction with the sequence ladder produced by chemical

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2 For comparison, random DNA cleavage is produced by a simple hydroxyl radical generator such as Fenton's reagent.

sequencing of the identical piece of DNA (D'Andrea et al., 1978; Takeshita et al., 1978). By the same technique, the dinucleotide sequence GA has been reported as a frequently cleaved site (Kross et al., 1982) The amount of cleavage is a function of the experimental conditions, e.g. amount of reduced iron or ionic strength. Possible modulation of the relative amounts of cleavag at the preferred sites will be discussed below.

The sequence 5' Pyr-G-C-Pur 3' has been suggested as a likely extended sequence for recognition by bleomycin (Mirabelli et al., 1982). However, there is still no definative evidence for the influence of neighboring bases, besides the proximate 5' G, on the location of single strand breaks by bleomycin. Mirabelli and co-workers tabulated the frequency of all four bases at positions neighboring 58 sites of bleomycin cleavage. The sequence 5' Pyr-G- occurred adjacent to the cleavage site 67% of the time (50% is random). However, the same data showed that10 residues away an A or C residue occurred 71% of the time. These numbers appear to be equally significant statistically, yet it is difficult to rationalize how the residues one complete turn of the helix away from the cleavage site can be important determinants of the specificity of a molecule the size of bleomycin. In addition, at sites catagorized as extensively cleaved by these researchers, both purine bases occurred at this position at least once. It seems clear that there is no absolute requirement for any sequence recognition by bleomycin beyond the preference for the previously mentioned dinucleotides.

Comparison of the cleavage specificity of several bleomycin congeners and analogues with respect to the differences in their structures sheds some light on the question of which substituents are responsible for the drugs specificity.

Phleomycin has a non-aromatic, non-planar ring, instead of the co-planar bithiazole rings of bleomycin (see figure 1). Phleomycin does not lengthen DNA in hydrodynamic experiments, and is not expected to be able to intercalate because of the non-planarity of its ring system. The similarity of the cleavage patterns generated by bleomycin-A2 and phleomycin suggest that intercalation is not essential for drug binding. It also indicates that the bithiazole moiety is not capable of imparting the observed sequence specificity independent of the rest of the molecule (Povirk et al, 1981 and Kross et al, 1982).

Tallysomycin differs only by the addition of a gulose sugar residue and a hydroxyl to the aminoethyl chain which connects the bithiazole to the methyl valerate of the bleomycinic acid (see *figure* 1). The pattern of cleavage of bleomycin-A2, B2, and phleomycin appears to be a subset of tallysomycin-A sites (Kross et al, 1982). The modifications to the aminoethyl chain differentiating Tallysomycin seem to diminish the sequence specificity of the drug.

Deglycobleomycin is produced by removal of the two sugar residues attached at the ß-hydroxyhistidine. This modification causes little change from the parent compound in either metal binding characteristics or the sequence specificity of DNA cleavage (Umezawa et al., 1984).

Kross and co-workers (1982) also investigated the ability of various bithiazole derivatives with different cationic side chains to compete with Fe(II)-bleomycin under DNA cleaving conditions (vide supra). The location and amount of cleavage by the drug was then assayed. The effectiveness of the competition was a function of the binding constant of the analogue. The binding constant

was a function of the number and spacing of the side chain's positively charged groups. Cleavage by bleomycin was diminished uniformly at all sites. These model compounds apparently give only non-specific competition to bleomycin binding. This indicates that the bithiazole + side chain by itself is not sufficient to impart to bleomycin its preference for cleaving DNA at certain sites.

The picture that emerges from the evidence presented above is consistent with the two functional domain concept only as a first approximation. Investigations with congeners and analogues of bleomycin demonstrate that bleomycin's interaction with DNA leading to cleavage of the strand is not quite so simple (vide supra). It is helpful to differentiate between non-competent or non-specific binding and competent binding, i.e. binding that allows the drug to cleave the DNA. The distinction is exemplified by the lack of correlation betweeen the fluorescence quenching results of Kasai and co-workers (1978) and the competition experiment results by Hori (1979). The non-competent or non-specific binding was characterized by the results of Chien et al (1977), and Kasai and co-workers, cited above. They demonstrated that most of the binding energy of metal free bleomycin is from the association of the bithiazole rings and the cationic side chain with DNA. However, the reactive metal-bleomycin complex binds DNA significantly better (Chang and Meares,1984). The NMR results of Antholine and co-workers (1981) suggest that certainly the environment of at least some other groups on the bleomycin-metal chelate are effected by the interaction with DNA. From the temperature dependence of the bithiazole interaction with DNA, and comparison of bleomycin analogues differing only at the putative DNA binding region, it seems clear that full intercalation of the bithiazole rings is not a necessary mode for competent binding. Electrostatic interactions are important for binding. They are

additionally important for the generation of double strand breaks as we will discuss in the following section. Bleomycin's preference for mixed sequence, double-stranded DNA containing guanine has been clearly demonstrated.

III. GENERATION OF DOUBLE STRAND BREAKS

Background

The abiltiy of bleomycin to produce double strand cleavage of DNA was demonstrated in the early papers describing the drug's effects on nucleic acids (Suzuki et al., 1970; Haidle, 1971; Diers et al., 1975). As mentioned above, the net damage by bleomycin is not directly correlated with the drug's cytotoxic efficacy (Hecht et al, 1985). Because double strand breaks are repaired more slowly than single strand breaks (vide supra), they may be more directly related to the cytotoxic, and hence, antitumor effect of the drug. The mechanism and specificity of DNA double strand breakage by bleomycin has been the major focus of our research.

Characterization of double strand breaks by bleomycin.

Several groups have reported a greater rate of bleomycin cleavage of supercoiled DNA (form I) than relaxed circular or linear DNA (forms II and III) (Povirk et al., 1979; Mirabelli et al., 1983). The torsional strain of the supercoiling produces transient stretches of non-base-paired DNA. An estimate of 6-12% of base-pairs melted due to supercoiling within the physiological range was made by Dougherty and Koller (1982) based on measurements of hyperchromicity. By itself, this would argue against preferential binding of form I vs form II and form III DNA by bleomycin which requires duplex DNA for binding. However, Povirk and co-workers (1979) argued that, analagous to classic intercalators like ethidium bromide (Waring, 1970), the bithiazole moiety of bleomycin at least partially intercalates, thereby releasing torsional strain and providing a driving force for increased binding. Mirabelli and co-workers speculated that some unusual structures may be induced to form, such as kinks,

by the supercoiling and that these could be preferential binding sites for the drug. These researchers found decreased cleavage specificity of form I DNA under conditions of limited cleavage per molecule. With more extensive cleavage, the specificity became similar to that seen for bleomycin cleavage of the 145 base-pair fragment. We reason, however, that G, C rich regions of the DNA will be the regions that tend to remain duplex in supercoiled DNA. As the less strongly base paired regions loop out with supercoiling, they provide fewer competing binding sites and a higher local concentration of the drug at the regions remaining duplex.3

The first question addressed in this area was whether double strand breaks were produced as the accumulation of random, single strand breaks. Extrachromosomal plasmid or viral, covalently closed circular, supercoiled DNA provides a convenient and sensitive substrate for the comparison of single strand breaks with double strand cleavage. Analytical methods, e.g. electrophoresis, and isopycnic centrifugation, are available that separate form I (covalently closed, supercoiled), formII (nicked circular, relaxed), and form III (linear) DNA. A single nick will relax the supercoiled DNA to give form II DNA. A second nick, near a nick on the opposite strand, will generate linear, form III DNA. Comparison of the rate of production of form II and form III DNA from form I DNA gives information on the relationship between the two types of damage caused by bleomycin.

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[3] It should be noted that experiments comparing these DNA forms are difficult to do quantitatively. As discussed in the following section, the bleomycin cleavage reaction is very sensitive to conditions. The proceedures used to prepare the three forms of plasmid DNA used in these experiments can introduce differences in such things as ionic strength and divalent metal concentration.

Freifelder and Trumbo (1969) derived an equation to calculate the maximum number of base pairs between nicks on opposite strands which cannot prevent double strand cleavage. The equation describes an ideal situation where the accumulation of random and independent single strand breaks produces double strand cleavages.4 Povirk and co-workers (1977) compared the rate of bleomycin cleavage of form I DNA to generate form II and form III DNA to the rate predicted by the random model of Freifelder and Trumbo. Double strand cleavage of supercoiled DNA by bleomycin occurred much more frequently than predicted from the random accumulation of single strand breaks. From these results it was suggested that double strand cleavages occur as single, or independent, events (Povirk et al., 1977).

The generation of double strand breaks as single, independent events implies the testable hypothesis that there is a recognition sequence specific for double strand cleavages. Restriction enzyme mapping experiments have been performed to test this possibility.5 It is possible to investigate the initial double strand cleavage reactions of bleomycin with supercoiled DNA as the substrate by quenching the reaction when full length linear (form III) DNA is produced.

Mapping of bleomycin generated double strand cleavage sites on PM2 circular DNA was attempted with the restriction enzymes Hind III and Hpa II (Lloyd et al., 1978). The electrophoresis assay shows a background of smeared DNA but distinct bands are also seen. The distinct bands in this assay cannot be generated from random single strand cleavage. However, the bands seen do not define the locations of specific double strand cleavage sites because they

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4 The equation is given in Results and Discussion.

5 A description of the technique is presented in *Appendix*. See also *Methods in Enzymology* **65** (1980).

do not add up to the full length DNA as required. A discussion of the criteria for establishing the validity of restriction enzyme mapping results is presented in the Results and Discussion section of this paper. At this point we note simply that no one has been able to propose a recognition sequence for double strand cleavage by bleomycin from restriction enzyme mapping results.

A different research group reported the nonspecific cleavage of supercoiled phiX 174 RF I DNA by bleomycin because they did not see distinct bands produced by the drug in their mapping experiments (Love et al., 1981). Under their conditions secondary cleavage by the restriction enzyme gave essentially a smear after electrophoresis. However, purification of the full length, bleomycin linearized DNA and then denaturation and electrophoresis did not show many internal nick sites.

Finally, the amount of single strand cleavage was compared to the amount of double strand cleavage for several bleomycin analogues using supercoiled DNA as the substrate (Huang et al., 1981). The deamido analogue, lacking the cationic N-terminal amide, produced very few double strand breaks although it was able to nick the form I DNA efficiently. The structure of the side chain was not critical, but the presence of a cationic group was required for the rapid generation of double strand breaks. The planarity of the bithiazole rings was also important for the specific generation of double strand breaks. Phleomycin has a reduced thiazole ring and cannot have co-planar rings. This congener had the proportion of double strand breaks selectively reduced relative to bleomycin-A2. Modifications of the C-terminal amine, in the metal binding domain, affected both single and double strand breaks similarly.

Approaches to the study of double strand cleavage by bleomycin

The proclivity of bleomycin for generating double strand breaks was the initial finding that aroused our curiosity. The data discussed above indicates that there must be an alternative mechanism(s) for the generation of double strand breaks beyond what is expected by the accumulation of independent single strand breaks. There are, in fact, two distinct questions: How is it that double strand breaks occur more rapidly than expected from the random accumulation of single strand breaks (Povirk and co-workers (1977 and 1979)? And, where are double strand breaks located on the DNA sequence, that is, is there any higher order cleavage specificity?

Initial mapping experiments were unable to define an extended recognition sequence. We took up the investigation to determine the non-random mechanism of double strand cleavage by bleomycin. Controled cleavage reactions were used to determine the relevant conditions for the generation of double strand breaks. We developed and applied a set of criteria for the use of the restriction mapping techniques for bleomycin cleavage.

Custom designed and synthesized oligonucleotides provide a means to ask precise questions about the interaction of drugs with DNA. We have used this approach to focus on how specific double strand breaks are produced by bleomycin. The use of palindromic sequences allowed us to effectively study both strands of the duplex in single experiments. Information on the influence of both the sequence and structure of the oligonucleotide on the cleavage reaction is obtained.

As mentioned above, although double strand breaks were proportional to single strand breaks, the ratio of their occurrence could be modified by conditions that seemed pertinent to the electrostatic interactions of the drug. With this clue and the results of our work on plasmid cleavage, restriction enzyme mapping and cleavage of palindromic oligonucleotides, we were able to formulate an alternative hypothesis for the mechanism of double strand cleavage: there is a positive cooperativity between the initial single strand breaks and subsequent single strand cleavage reactions in the same region on the DNA that promotes the formation of double strand cleavages. Several experimental systems where devised to test this hypothesis. The results provide an explanation for the non-random generation of double strand breaks by bleomycin.

RESULTS AND DISCUSSION

Specificity of double strand cleavage of plasmid DNA

It is necessary to define the term "specificity" operationally to discuss our results. Agarose gel electrophoresis was used to assay the cleavage of plasmid DNA. The resolution of these agarose gels, however, is not sufficient to separate DNA fragments differing by less than 100 to 200 base-pairs in the size range between approximately 1000 base-pairs and full length pBR322 (4362 base pairs). If we consider only sequence recognition as the determining factor for the number of times a piece of DNA is cut, then discrete bands would be produced, assuming a random base sequence, from cleavage at a recognition sequence of 4 or more bases.[1,2] A shorter recognition sequence would result in fragments too close in size to be resolved. The restriction enzyme mapping technique is described in *Methods*. We note here that the same resolution factor is encountered when the restriction enzyme digest products are analyzed by agarose electrophoresis

In the *Introduction*, we discussed published reports of the preferred sequences at which bleomycin generated single strand breaks (D'Andrea et al., 1978; Takeshita et al., 1978; Kross et al., 1982). From these reports, it is generally agreed that 5'G-(C,T, or A) 3' are potential cleavage sites. If we consider all such sites in the pBR322 DNA used for our experiments, the generation of double strand cleavages by the random accumulation of single strand breaks would generate a family of fragments of random and diverse sizes with respect

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[1] The length of random sequence required to have a high probability of containing a recognition sequence is $1/(.25)^n$, where n is the number of bases in the recognition sequence.

[2] Compositional fluctuations do occur in natural DNA (Crothers, 1969). For example, within any stretch of DNA there can occur more than the expected 50% of G:C base pairs. These could allow discrete bands to be seen with less than the expected recognition sequence.

to the resolution of the agarose gel electrophoresis assay. Therefore, a smear would result if this was the sole mechanism of double strand cleavage by bleomycin. Our initial results are consistent with previous reports and indicate that there must be an additional mechanism for double strand cleavage.

The results of cleavage of linear pBR322 DNA by bleomycin as a function of Fe(II) concentration can be seen in *figure* 5. An excess of Fe(II)-bleomycin results in a smear of DNA fragments. The ionic strength was varied in this experiment also. In agreement with previous findings that electrostatic interactions were important for bleomycin binding, it can be seen, in lanes 1-5 vs lanes 6-10, that more cleavage occurs at the lower ionic strength (vide supra). At each ionic strength, the amount of cleavage increases porportionally with the concentration of reduced iron, as expected. Note that the full length DNA remaining has probably suffered single strand breaks, although no double strand breaks have yet occurred. The background of smeared reaction products indicates that there is a random accumulation of single strand breaks generating double strand breaks. However, when the amount of Fe(II)-bleomycin was limited, some discrete bands were resolved. This indicates there must be an additional mechanism for producing double strand breaks in linear DNA besides the accumulation of random single strand breaks.

Figure * 5

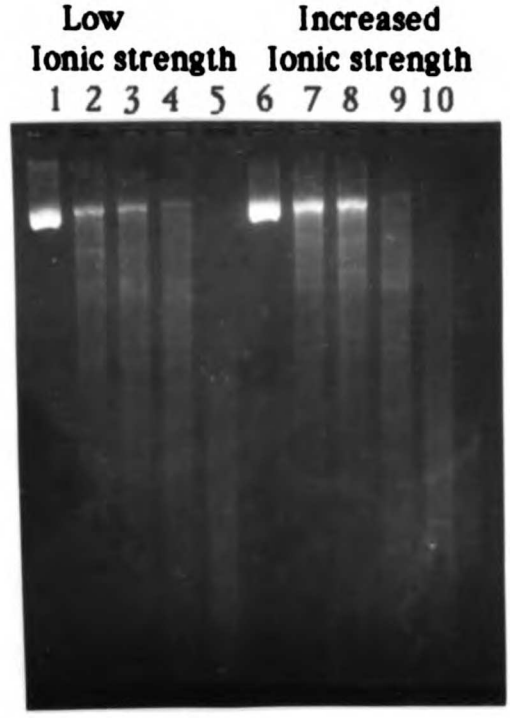

Figure 5. Cleavage of linear DNA as a function of ionic strength and [Fe(II)]. Purified Eco R1 linearized pBR322, ~1.8 x 10^{-8} M, was brought up in a buffer containing10 mM Tris (pH 7.5), 10 mM $MgCl_2$, and 1 mM DTT, and no additional salt (low salt buffer) for lanes 1-5, or 50 mM NaCl (medium salt buffer) lanes 6-10. The concentration of bleomycin was 8.8 x 10^{-7} M. The Fe(II)/BLM ratio was as follows: 0.1 in lanes 2 and 7; 0.2 in lanes 3 and 8; 0.5 in lanes 4 and 9; and 1.7 in lanes 5 and 10. The final reaction volume was 11 ul, samples were incubated for 20' on ice, then quenched with 1 ul 0.4M EDTA. Samples were analyzed by 1.4% agarose electrophoresis, equilibrated with ethidium bromide and photographed under UV light.

Gels with an agarose concentration of1-1.8% easily separate supercoiled (covalently closed circular) form I DNA, nicked (relaxed circular) form II and linear form III DNA. A single nick is sufficient to relax form I DNA to form II, and full length form III DNA is generated by a single double strand break. A limited reaction with Fe(II)-bleomycin produces a mixture of all three forms. The proportion of each is dependent on the extent of the reaction. Thus, bleomycin's reaction with supercoiled DNA can be controlled so that a significant proportion of the DNA receieves only one double strand cleavage per molecule. This allows investigation of the initial double strand cleavage generated by bleomycin. It is presumed that any double strand cleavage specificity greater than that seen for single strand breaks would be manifested in the initial double strand break.

An example of this type of experiment is seen in *figure* 6. Bleomycin cleavage of form I plasmid DNA produces one, double strand cleavage rapidly under conditions where an appreciable amount of the substrate DNA remains uncut (lanes 12 and 13). From the results of the 5 minute reaction (lane 13), we estimate the fraction of uncut plasmid (f_I) to be 0.05 and the fraction of form III (f_{III}) to be 0.1. The number of single strand breaks (n_1) can be calculated from the fraction of uncut plasmid and the number of double strand breaks (n_2):

$$f_I = \exp-(n_1 + n_2)$$

In this experiment, n_2 is approximately 0.1.[3] Therefore since f_I is 0.05, the average number of single strand breaks per molecule is calculated to be 2.9.

[3] Note that n_2 is equal to f_{III} since each full length linear molecule has recieved only one double strand break.

We used the equation developed by Freifelder and Trumbo (1969), which models the production of double strand cleavage by a random accumulation of single strand breaks, to compare bleomycin's activity with the random mechanism.

$$h = [L(1 - f_I^*)/2n_1] - 1/2$$

where

h = maximum number of base pairs necessary to prevent a double strand break.

L = number of possible cleavage sites per strand.

n_1 = the number of actual single strand breaks per strand.

f_I = fraction of molecules without a double strand cleavage, remaining form I.

$f_I^* = f_I \exp(1/n_1)$

Their equation assumes $h \ll L$, all sites are equivalent, a random base sequence, there is no overlap between sites able to yield a double strand break, and the number of nicks on each strand are equal. We used the values h = 15, which is appropriate for the electrophoresis assay conditions, and L = 4362, the number of base-pairs contributing to the duplex stability. If we use the value of $n_1 = 2.9$ calculated from the data, the expected value for the fraction of uncut form I DNA (f_I) from the random model for generating double strand breaks is calculated to be 0.94. This is significantly different from the actual experimental result of $f_I = 0.05$, and provides strong support for the hypothesis that an additional mechanism for producing double strand breaks, besides the accumulation of random single strand breaks, is required to explain the data.[4]

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[4] Alternatively, one can use the same equation with the experimental value for f_I and calculate the value of n_1 expected from the random mechanism: if f_I is 0.05, n_1 is predicted to be 20 instead of 2.9 as calculated from the data.

Figure * 6

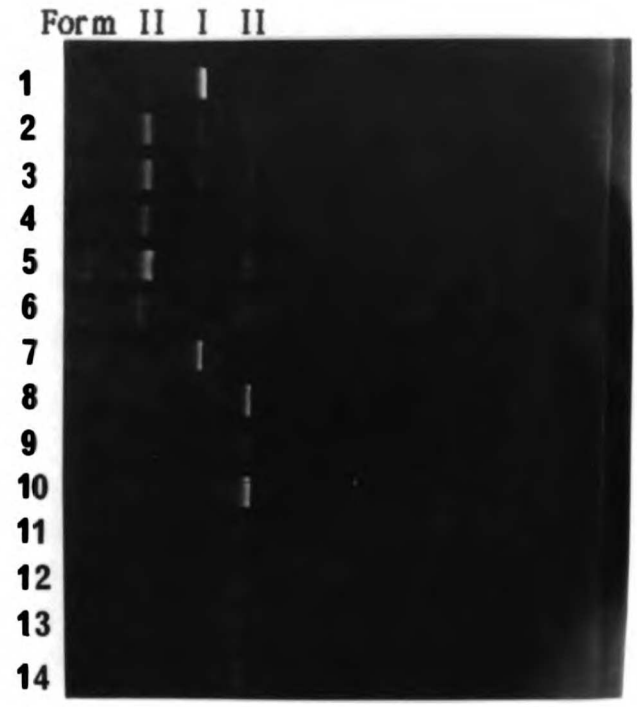

Figure 6. A total reaction volume of 30 ul was incubated on ice. Bleomycin (blm) was 5 x 10^{-7} M, Fe(II) was 5 X the bleomycin concentration, supercoiled, form I or linear, form III pBR322 was1 x 10^{-7} M, the buffer contained 50 mM NaCl, 10 mM Tris (pH 7.5), 10 mM $MgCl_2$, and 1 mM dithiothreitol (DTT). Aliquots were removed and quenched with an excess of EDTA at the time points listed. Samples were analyzed by 1.4% agarose electrophoresis, equilibrated with ethidium bromide and photographed under UV light:
Lane1, form I DNA marker.
Reaction of bleomycin with form I DNA was for: 2) 5', 3)15', 4) 30', 5) 1 hr, 6) 5.5 hr, 7) control, 1 hr with Fe(II), no bleomycin.
Lane 8, form III DNA marker.
Reaction of bleomycin with form III DNA was for: 9) 5', 10) 15', 11) 30', 12) 1 hr, 13) 5.5 hr, 14) control, 1 hr with Fe(II), no bleomycin.

It is important to note that under the same conditions used to rapidly generate one double strand cleavage of supercoiled DNA, bleomycin did not generate smaller fragments via sequence specific cleavage of the full length plasmid. The sequences, of course, are the same for the different forms. However, it must be remembered that one double strand break anywhere on the plasmid will produce the form III product, whereas the location of the double strand break must be specific for a detectable band to be produced by the assay used. Therefore, these data indicate only that double strand breaks occur more rapidly with supercoiled DNA as the substrate than with linear DNA. They do not require a higher order of sequence specificity than has been described above.

The torsional strain of the supercoiling produces transient stretches of non-base-paired DNA. An estimate of 6-12% base-pairs melted was made by Dougherty and Koller (1982) from measurements of hyperchromicity. By itself, this would argue against preferential binding of bleomycin to form I vs. form II or form III DNA because the drug requires duplex DNA (vide supra). However, Povirk and co-workers (1979) argue that, analagous to classic intercalators like ethidium bromide (Waring, 1970), the bithiazole rings can intercalate. Intercalation removes negative supercoils and relieves torsional strain, thereby providing a driving force for initially stronger binding to form I DNA. As discussed above, experiments with analogues of the DNA binding portion of bleomycin do support intercalation by the bithiazole rings. Nonetheless, the data pertinent to intercalation as the mode of binding of the biologically active metal complex is not entirely convincing. In addition, other modes of binding can unwind the DNA and presumably gain the same decrease in free energy of binding (for example, the steroid amine irehdiamine, Waring, 1970)

An alternative suggestion has been made that some unusual structures may be induced to form, e.g. kinks, by supercoiling and that these may be preferential binding sites for the drug (Mirabelli et al., 1983). Although no permanently open areas are expected, supercoiling is expected to cause transient base-pair opening (Vologodskii et al, 1979, Hsieh and Wang, 1975, and Jacob et al, 1974). Given the requirements of bleomycin for duplex DNA for binding, as discussed above, we suggest that transiently melted regions and unusual structures decrease the number of competing binding sites. This amounts to an effectively higher bleomycin concentration and a higher rate of cleavage.

The existance of a "high affinity", canonical recognition sequence causing preferred locations for double strand cleavage by bleomycin would be amenable to determination by the restriction enzyme mapping technique (see *Appendix*). Several criteria must be strictly met to be assured that the double strand cleavage at a particular location is due to recognition of the sequence and not some other factor(s). The criteria we have developed follow. Each cleavage by a restriction enzyme having a single recognition site will generate two fragments for each sequence specific double strand cleavage by the drug. The lengths of the pairs of fragments produced by the double digest must add up to the full length of the DNA substrate. The sites located using one restriction enzyme should be confirmed by any other restriction enzyme used to map the drug's specificity. Finally, restriction enzymes that cleave a given piece of DNA at approximately the same site should give identical cleavage patterns.

As mentioned in the *Iintroduction*, mapping of the sites of double strand cleavage by bleomycin with restriction enzymes has been reported by several groups (Lloyd et al., 1978; Love et al., 1981; Mirabeli et al., 1979). These

straightforward attempts have not succeeded in meeting the criteria mentioned above (vide infra).

Figure 7 is an example of a simple mapping experiment (see *Methods*). Isolated and purified full length DNA, generated by one bleomycin-directed double strand cleavage, is used for the experiment. Each distinct double strand cleavage site generates two fragments after the restriction enzyme digestion. As mentioned above, if sequence recognition is the only factor imparting specificity, the resolution of the gel is such that a 4 base recognition site would be expected for the discrete bands. However, we were not able to satisfy the criteria presented above for a self-consistent set of double strand cleavage locations from these patterns of bands for the following reasons. The restriction enzyme digest was incomplete, since most of the DNA remained full length. There was a large amount of background smear (random double strand cleavage). And finally, the discrete bands do not add pair-wise to the full length plasmid.

Figure * 7

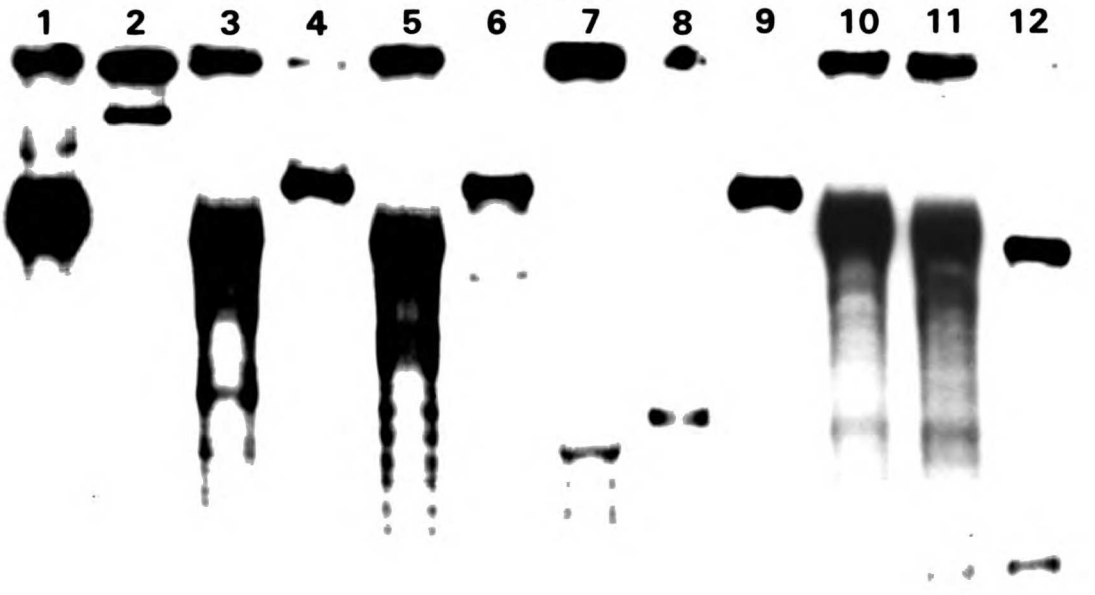

Figure 7. Restriction enzyme mapping. Form I pBR322 was cleaved by Fe(II)-bleomycin, (BLM), at a ratio of 20/1 drug to DNA molecules. Buffer contained10 mM Tris (pH 7.6) with 25 mM ß-mercaptoethanol. The full length linearized DNA generated by the cleavage reaction was purified by low melting temperature (LMT) agarose electrophoresis (see Appendix). The purified product was labelled at the 5' end with ^{32}P by T4 polynucleotide kinase. The labelled, bleomycin linearized pBR322, was then digested with a restriction enzyme as indicated below. The controls, without bleomycin reaction, used pBR322 labelled with ^{32}P by nick translation. The products were analyzed by 1.8% agarose electrophoresis and autoradiography.

1) purified bleomycin generated linear pBR322;
2) uncut pBR322;
3) BLM/PvuII;
4) PvuII alone;
5) BLM/EcoRI;
6) EcoR1 alone + marker fragments from partial cleavage of EcoR1 by HinfII
7) BLM/HinfI;
8) HinfI alone;
9) SalI alone;
10) BLM/SalI;
11) BLM/HincII;
12) HincII alone.

A simple way of testing the validity of restriction enzyme mapping of bleomycin canonical cleavage sites was performed using two enzymes that cleave pBR322 at approximately the same location. As mentioned above, digest of full length DNA generated by specific double strand cleavage by such enzymes should produce identical patterns of bands. Eco R1 and Hind III restriction enzymes cleave pBR322 at sites only 31 base-pairs apart, a difference that cannot be resolved by agarose gel electrophoresis except for the smaller fragments that might be generated by a double digest experiment. As in the previous experiment, the purified full length linear DNA generated by the initial double strand cleavage of form I pBR322 by bleomycin was used as the substrate for restriction enzyme digestion. Cleavage by the drug at specific, double strand cleavage recognition sequences will produce identical cleavage patterns after digestion with these two enzymes.

The results are shown in *figure* 8. As in the previous experiment, the incompleteness of the enzyme digest and the random double strand cleavage make analysis difficult. However, discrete bands are resolved. Many of these bands are the same for the two enzymes, but a significant number of bands do not match (lanes 4 and 5). This anomolous result poses a problem for a simple interpretation of the mapping experiments. The presence of several "high affinity" sites remains possible, but it is weakened by the non-matching bands from the Eco R I and Hind III digestions. Discrete bands are also resolved after digestion of the bleomycin cleavage product with Ava I and Hinf I restriction enzymes. The results were similar. The large number of bands and the lack of completeness of the enzyme digestion preclude the successful mapping of the pattern of bands to specific double strand cleavage sites.

Figure * 8

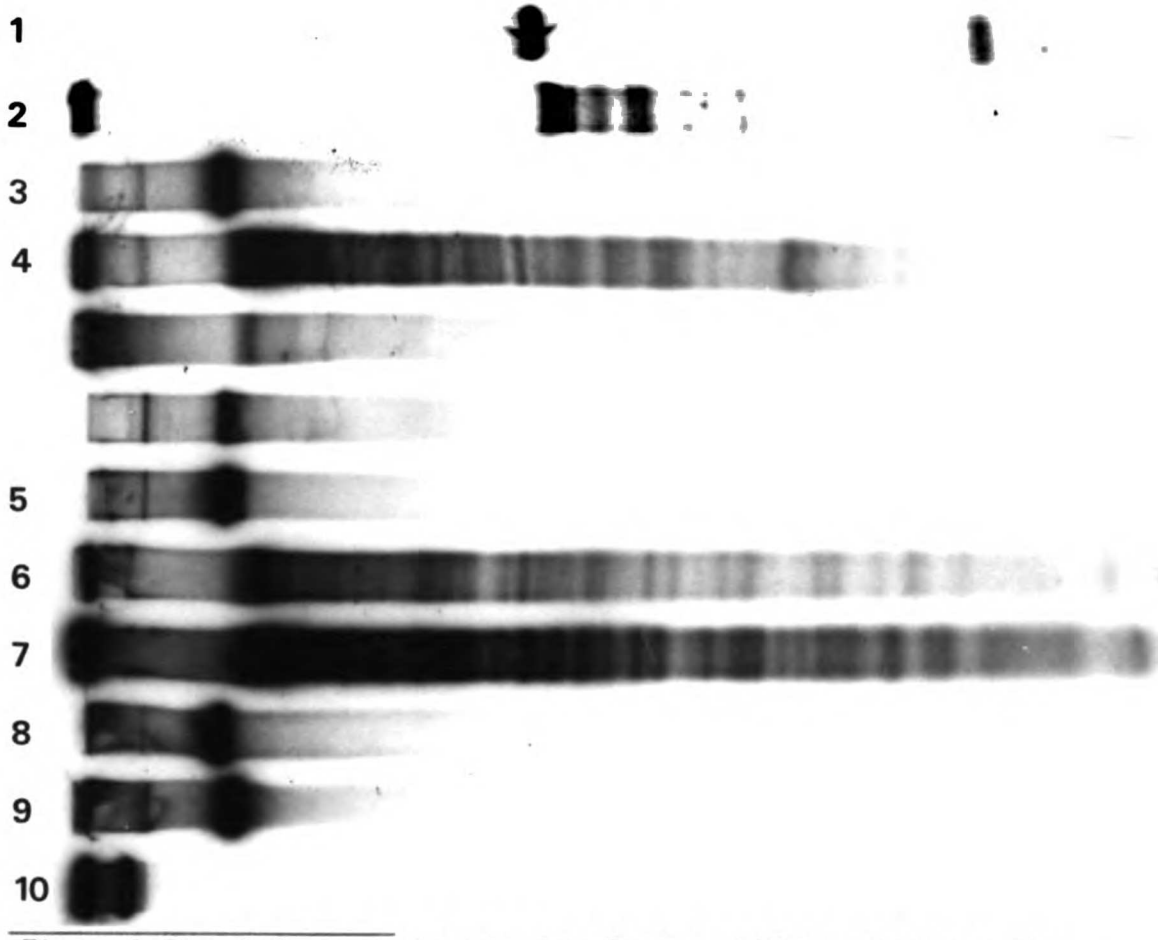

Figure 8. Restriction enzyme mapping. Form I pBR322 was cleaved by Fe(II)-bleomycin, (BLM), at a ratio of 20/1 drug to DNA molecules. Buffer contained10 mM Tris (pH 7.6) with 25 mM ß-mercaptoethanol. The full length linearized DNA generated by the cleavage reaction was purified by low melting temperature (LMT) agarose electrophoresis (see Appendix). The purified product was labelled at the 5' end with ^{32}P by T4 polynucleotide kinase. The labelled, bleomycin linearized pBR322, was then digested with a restriction enzyme as indicated below. The controls, without bleomycin reaction, used pBR322 labelled with ^{32}P by nick translation. The products were analyzed by 1.8% agarose electrophoresis and autoradiography.

1) HinfI alone;
2) BLM/HinfI;
3) AvaI alone;
4) BLM/AvaI;
5) HindIII alone;
6) BLM/HindIII;
7) BLM/ EcoR1;
8) EcoR1 alone;
9) purified bleomycin generated linear pBR322
10) uncut pBR322.

We were concerned that the non-matching bands from the Eco R1 and Hind III mapping experiment could be due to contaminating, non-pBR322 DNA. Small amounts of chromosomal DNA fragments, if carried through the purification process, could produce such an effect. We designed the following experiment to check for the presence of competing DNA and to allow mapping of only the pBR322 DNA. *Figure* 9 demonstrates the results of mapping experiments performed as above, but with an additional digestion with several restriction enzymes which do not cut pBR322. These enzymes are expected to digest any contaminating E. Coli DNA into smaller fragments, thereby clearing the lanes of any non-pBR322 contaminating DNA. Lanes 7 and 8 demonstrate that the pBR322 used in these experiments did not have any significant amounts of contaminating DNA. The pattern of bands produced by restriction enzyme mapping with prior digestion by non-pBR322 cutters is the same as the pattern generated without this prior digestion. Therefore, the non-matching bands cannot be explained as being due to contaminating DNA.

Figure * 9

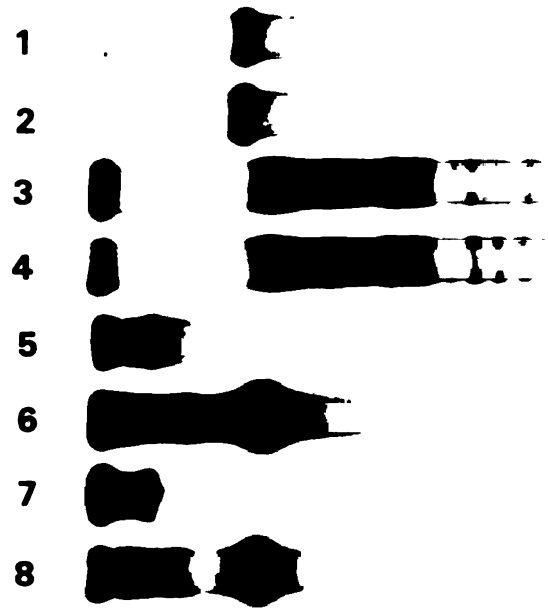

**Figure* 9. Restriction enzyme mapping with pre-digestion by non-pBR322 cutters (see text). Form I pBR322 was cleaved by Fe(II)-bleomycin at a ratio of 20/1 drug to DNA molecule. The buffer contained 10 mM Tris (pH 7.6) with 25 mM β-mercaptoethanol. The full length linearized DNA generated by the cleavage reaction was purified by LMT agarose electrophoresis. The purified product was labelled at the 5' end with ^{32}P. The labelled, bleomycin linearized pBR322 was then digested with the restriction enzymes indicated. The controls, without bleomycin reaction, used pBR322 labelled with ^{32}P by nick translation (lanes 2, 4, 7, and 8). The products of this second digestion were analyzed by 1.8% agarose electrophoresis and autoradiography.

1) Nick translation labelled PBR322/SacI/KpnI/HindIII;
2) Nick translation labelled PBR322/SacI/KpnI/EcoRI;
3) BLM//SacI/KpnI/HindIII;
4) BLM/SacI/KpnI/ EcoRI;
5) SacI and KpnI alone;
6) BLM/SacI/KpnI;
7) purified bleomycin generated linear pBR322;
8) uncut pBR322.

As described in *Methods*, the bleomycin generated ends were labelled with ^{32}P using polynucleotide kinase before the restriction enzyme digestion. It was possible that the structure of the ends produced by the bleomycin reaction prevented the uniform labelling of the ends by polynucleotide kinase. We therefore changed the protocol of the mapping experiment so that the 3' ends generated by digestion with the restriction enzymes were the labelled ends rather than the drug generated ends.[5] The results of this modification of the experiment are shown in *figure* 10. The results are quite different from our previous mapping experiments. Using this labelling procedure, the electrophoresis assay detects diffuse clusters of bands instead of discrete bands over a smeared background. This is not consistant with the presence of a canonical recognition sequence as the major mechanism of double strand cleavage by bleomycin. The results of this experiment suggest the location of the double strand breaks is no more specific than the single strand breaks. That is, an initial single strand break determines the location of the subsequently generated double strand break. This also supports the hypothesis that a factor other than sequence specificity is responsible for the rapid production of double strand breaks by bleomycin.

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[5] T4 DNA polymerase was used to label the digested DNA with [alpha-^{32}P] NTPs. This enzyme has 5' —> 3' polymerase activity. It does not appreciably label bleomycin generated damage (Niwa and Moses, 1981; and our results).

Figure " 10

Figure 10. Restriction enzyme mapping, modified labelling procedure (see text). Form I pBR322 was cleaved by Fe(II)-bleomycin at a ratio of 20/1 drug to DNA molecules in 10 mM Tris (pH 7.6), 0.1 mM $CaCl_2$, and 25 mM ß-mercaptoethanol. The full length linearized DNA generated by the cleavage reaction was purified by LMT agarose electrophoresis. The purified product was incubated with either Eco R1 or Hind III as indicated. The DNA was precipitated, brought up in the appropriate buffer, and labelled at the restriction enzyme generated ends by reaction with T4 DNA polymerase and [alpha ^{32}P]-NTPs. The labelled products were then analyzed by 1.8% agarose electrophoresis and autoradiography:
1) control, nick translation labelled pBR322;
2) Hind III, no bleomycin reaction;
3) Hinf I, no bleomycin reaction;
4) Bleomycin linearized DNA/Hind III;
5) Bleomycin linearized DNA/Eco R1;
6) Bleomycin linearized DNA, no restriction enzyme reaction.

In addition to the lack of completeness of the restriction enzyme digests, we noticed that the bleomycin-generated "full length" DNA migrates equivalently with the actual full length, linear DNA when a very limited reaction is allowed to occur. If the reaction is allowed to continue, the linearized DNA is shortened by the drug well before secondary bands begin to appear. This can be seen as a slight shift in mobility for the bleomycin generated full length DNA relative to the enzymatically generated full length DNA in *figures* 7, 8, and 9. This suggested to us the possibility of bleomycin "chewing in" from the ends at the double strand cleavage sites. This hypothesis will be discussed further.

The number of single strand breaks per molecule generated in the experiment described in *figure* 6 was calculated to be approximately 3. This assumes all cleavage reactions are completely independent. If this estimate is reasonable, then several observations from the experiments described above require explanation. The first is the lack of completeness of the restriction enzyme digestions. Bleomycin damaged regions may be refractory to cleavage by the enzymes. This could indicate that many more than 3 nicks have occurred. Alternatively, bleomycin-damaged DNA is known to inhibit DNA dependent enzymes (vide supra). The restriction enzymes could interact with the damaged sites. If the interaction was strong enough, only a few sites per molecule would be required to accomplish sufficient inhibition to account for the result.

The following observations seem to be related to the incompleteness of the restriction enzyme digest. Although discrete bands can be seen when bleomycin-damaged ends are labelled and then the DNA is cut with restriction enzymes, many of the bands produced by mapping with Eco R1 and Hind III do not match. There are fewer discrete bands detected when the restriction

enzyme derived ends are labelled instead of the bleomycin generated ends. These results appear to be manifestations of inhibition, to varying degrees, of the enzymes by bleomycin generated damage.

The last observation that is difficult to explain by a simple recognition sequence hypothesis is that the linear DNA isolated after bleomycin reaction is slightly smaller than the full length DNA generated by restriction enzyme cleavage. As mentioned above, this could be caused by the "chewing in" or clustering of cleavage sites near the initial cleavage locations. This seems to imply more damage than predicted above, although the damage may be clustered in only a few locations, with subsequent cleavage occurring cooperatively in the same area as the initial damage.

We conclude, from these results, that the sequence specificity of bleomycin and the rate at which double strand breaks occur are distinct and separate problems. The location of the initial single strand breaks produced by bleomycin reaction with form I DNA may be modulated as a function of the supercoiling. Subsequent cleavage after initial damage occurs cooperatively in the same regions of the DNA. DNA dependent enzymes, like polynucleotide kinase and restriction enzymes, are inhibited by the bleomycin-damaged DNA. In the case of restriction enzymes, Eco R1 and Hind III are affected differently by the bleomycin generated damage. The generation of double strand breaks by bleomycin beyond what can be accounted for by the random accumulation of single strand breaks is not due to the existence of any higher affinity sequences causing double strand cleavage as a single event. Rather, one must hypothesize an alternative factor causing a relative increase of cleavage

opposite a previously generated single strand break leading to the rapid generation of double strand breaks.

Cleavage of oligonucleotides with specified sequences

We designed several palindromic oligonucleotides to investigate the details of double strand cleavage by bleomycin. We also used these oligonucleotides to study the sequence specificity and time course of alkali labile damage by the drug. The use of palindromic oligonucleotides allows, in effect, the simultaneous study of both strands of the duplex, since the sequence is identical in the two directions. Our main goal was to gain insight into the mechanism by which non-random double strand breaks are generated by bleomycin.

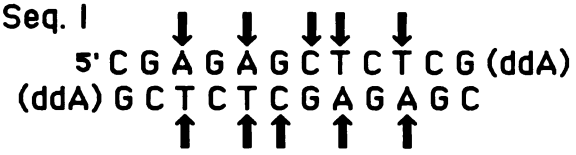
The frequency of single strand cleavage at specific sequences and the influence of neighboring bases on this frequency has been investigated (vide supra). The two oligonucleotides used in the following experiments contain several variations of sequences neighboring the 5' G-(C, T, or A) 3' cleavage sites (underlined), as shown below. These allow us to test the influence of neighboring bases on cleavage at preferred sites.

```
                PUR ..... PUR
              PYR ..... PUR
I.    5' C-G-A-G-A-G-C-T-C-T-C-G 3'
                      PUR ..... PYR

                   PYR ..... PUR
II.   5' A-T-G-A-A-G-C-T-T-C-A-T 3'
                      PUR ..... PYR
```

The following diagram summarizes the bleomycin cleavage results from *figure* 11. Cleavage locations are marked by arrows.

Seq. I


5' C G A G A G C T C T C G (ddA)
(ddA) G C T C T C G A G A G C

Seq. II

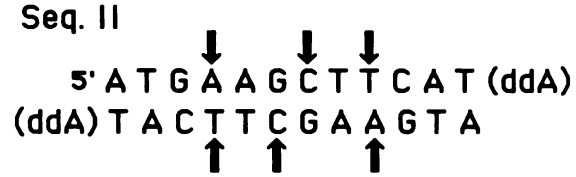

5' A T G A A G C T T C A T (ddA)
(ddA) T A C T T C G A A G T A

Figure ° 11

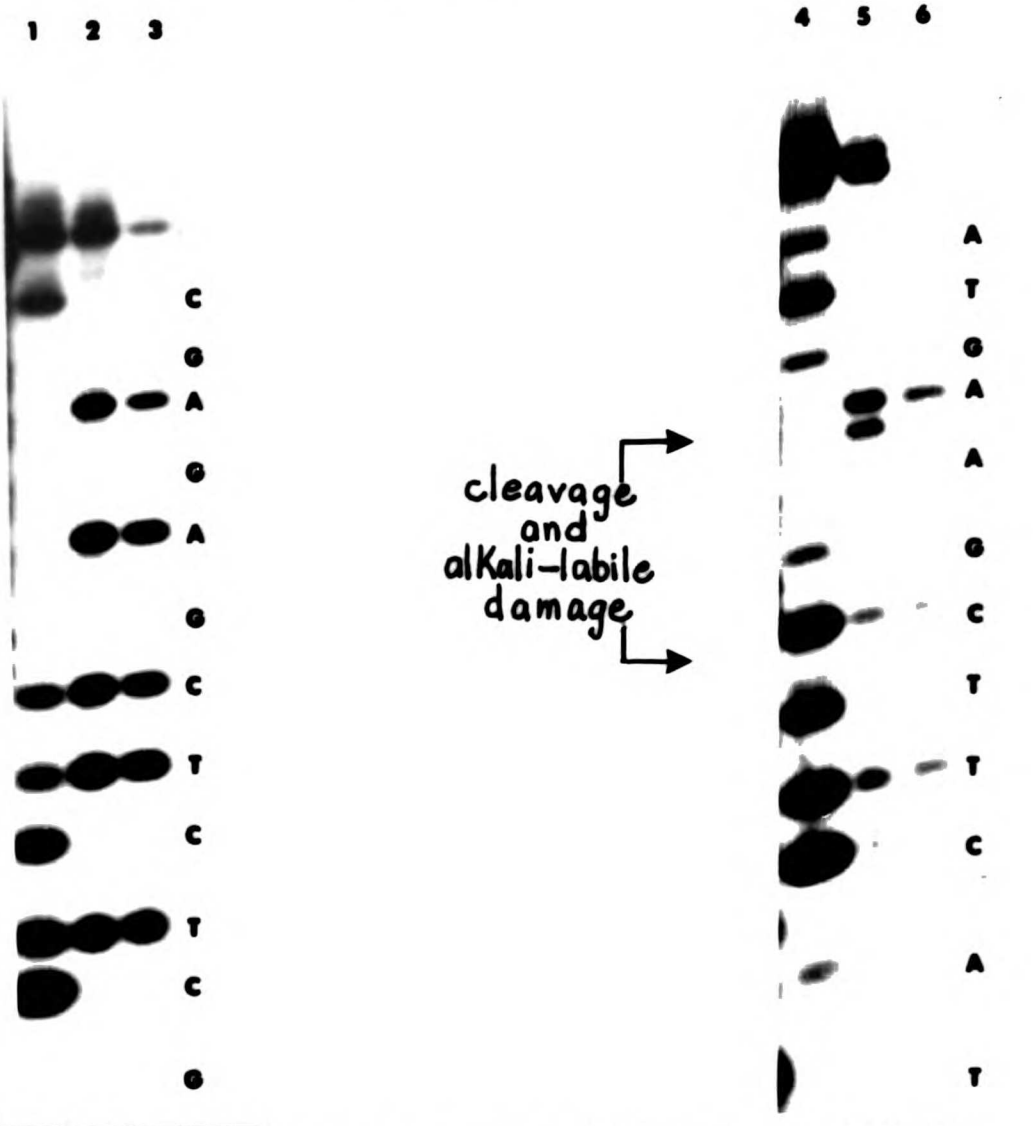


Figure 11. Sequence specific cleavage and alkali-labile damage of 3' [^{32}P]-dideoxyadenosine labelled oligonucleotides. Incubation of the end-labelled oligonucleotides with 40 uM bleomycin, 80 uM Fe(II) was for 10' on ice in 10 mM Na-phosphate, 5 mM dithiothreitol buffer. Samples were assayed by analytical 20% PAGE. Chemical sequencing markers of the oligonucleotide were run alongside (Maxam and Gilbert, 1980). The appropriate sequence is given to the right of each autoradiograph.

1) C + T sequence markers
2) Bleomycin/oligonucleotide reaction
3) same reaction + alkali treatment
4) C + T sequence markers
5) Bleomycin/oligonucleotide reaction
6) same reaction + alkali

As mentioned above, the sequences identified in the literature as most frequently cleaved single strand sites are (5'—>3') G–C, G–T, and G–A. The suggestion by Mirabelli et al (1982) that double strand cleavages occur preferentially at 5'pyr-G-C-pur3' sequences was based on the occurrance of these nearest neighbor sequences at cleavage sites more frequently than other sequences. In their results, however, all combinations of nearest neighbors were seen at sites that were extensively cleaved by bleomycin. Our results demonstrate that for a given stretch of DNA, bleomycin effectively cleaves the preferred dinucleotide sequences regardless of the neighboring sequences. Furthermore, unless an extended sequence promotes stronger binding of the drug to its DNA substrate, it does not seem likely that nearest neighbor sequences can be significant for the mechanism of double strand cleavage. To date there is no indication of any binding effects beyond that of the preferrred dinucleotide sequences (vide supra). Additionally, a short palindromic target sequence does not increase the likelihood of double strand cleavage to any appreciable degree under the usual conditions of ionic strength and temperature. For example, the stretch of duplex DNA within which single strand breaks on opposite strands would produce a double strand break was 15 base-pairs under the conditions used for the electrophoresis assays, while it is probable that a 5'G-(C, T, or A)3' sequence will occur once in every 5 or 6 bases. We conclude that sequence effects do not promote double strand cleavage beyond that expected from the random accumulation of single strand breaks.

Charge effects: End cleavage and Cross strand cleavage

Figure 12 demonstrates the results of a similar experiment using sequence I labelled at the 5' terminus. Again there is cleavage at the three expected

preferred cleavage sites. There are two additional damage sites that seem to be correlated with the presence of the 5' terminal phosphate used for labelling the oligonucleotide. There are bands that appear to correspond to the C-G of the 3' terminal sequence and a very intense band for a fragment apparently shorter than that caused by cleavage at the terminal 5' G-A 3' sequence. Our best guess is that this band is due to bleomycin cleavage at the terminal 5' $=P^{32}C$. Its mobility probably is anomalous because of a salt effect on the electrophoresis. We note that these unusual sequences are damaged even at the shortest reaction times when most of the oligonucleotide is uncut and full length. These last two locations were not damaged in the previous experiment in which an uncharged label was attached to the 3' terminus, nor was there cleavage at unusual sequences near the uncharged 3' terminus. We conclude that the 5' terminal phosphate affected the location of cleavage by bleomycin and promoted cleavage near the phosphorylated terminus. This is consistent with the shortening of bleomycin-generated full length DNA with increasing reaction time, as reported above. It is also consistent with the hypothesis that increasing the negative charge of locations on the oligonucleotide promote cleavage by bleomycin near the locus of charge.

Figure ° 12

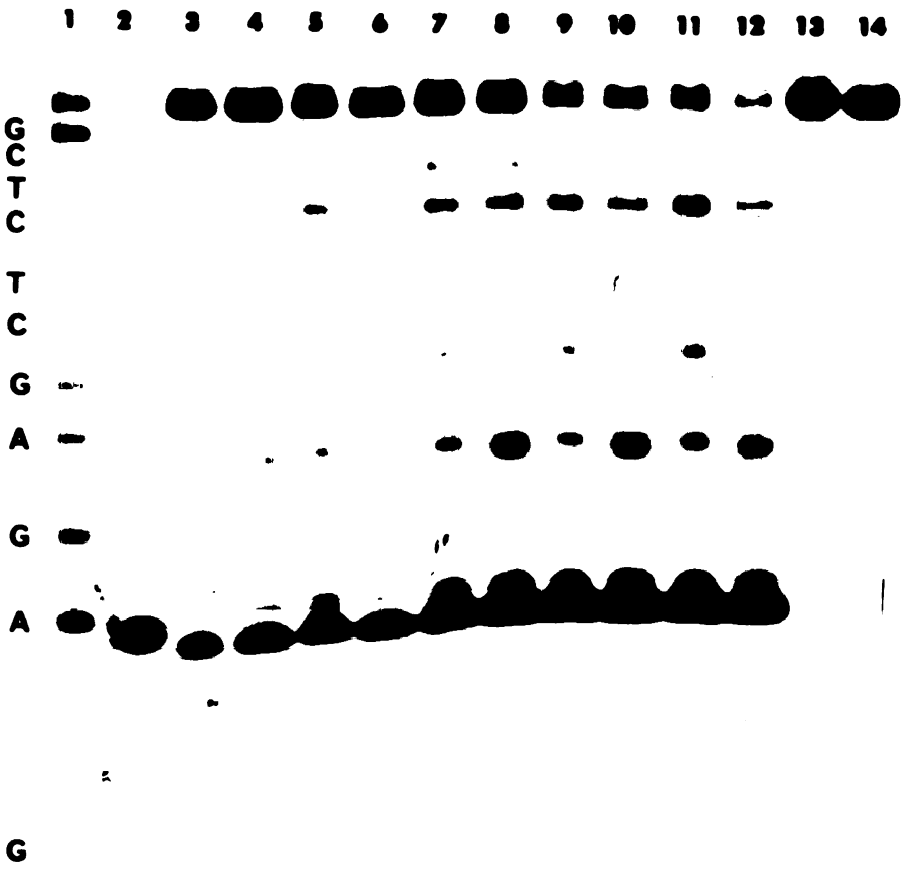


Figure 12. Bleomycin cleavage of the 5'(^{32}P) labelled dodecamer. Reaction, in 10 mM Na phosphate buffer (pH 7.6) on ice, of 1.6 mM Fe(II)-bleomycin with nanomolar amounts of oligonucleotide for various lengths of time, with or without subsequent alkali treatment of the digestion products. Samples were assayed by analytical 20% PAGE. Chemical sequencing markers of the oligonucleotide were run alongside (Maxam and Gilbert, 1980). An estimate of the location of bleomycin fragments is given to the right of the autoradiograph.

1) G+A sequence markers
2) Snake venom phosphodiesterase partial digest reaction products
3) 1.5 min. reaction 4) reaction + alkali treatment
5) 2.5 min. reaction 6) reaction + alkali treatment
7) 5.0 min. reaction 8) reaction + alkali treatment
9) 10.0 min. reaction 10) reaction + alkali treatment
11) 30.0 min. reaction 12) reaction + alkali treatment
13) 30.0 min. control, (-) bleomycin 14) 30.0 min. control, (-) Fe(II)

In addition to cleavage at all occurrences of the expected sites, the sequence (5'—>3') C–I–C was a major location on both the 3' and 5' end labelled oligonucleotides (*figures* 11 & 12). It is noteworthy that this cleavage site is complementary to the preferred site (5'—>3') G–A. This sequence was consistently cleaved under conditions where there was still uncut (full length) oligonucleotide available to the drug. Cleavage at 5'CT has been reported before, but it is not known as a preferred site (Mirabelli et al,1982).

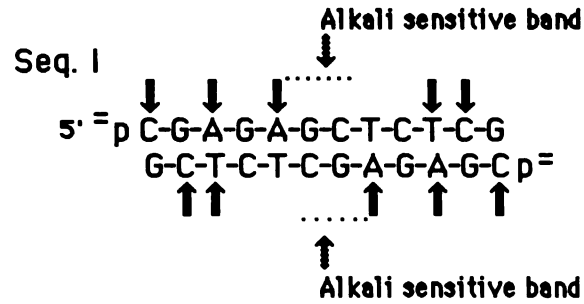
These results suggests the following alternative to sequence recognition as the mechanism of non-random double strand cleavage. An initial single strand cleavage by bleomycin generates a locus of increased negative charge on both sides of the excised nucleotide. Therefore, the nick site could provide an increased electrostatic interaction with the cationic drug molecule. This could promote cleavage on the same strand, i.e. a pseudoprocessive chewing in from the end. A double strand break would result from this cooperativity when a single strand break was generated near a nick on the opposite strand. Alternatively, direct production of a double strand break would result if binding at the nick promoted cleavage of the opposite strand. We report the results of experiments designed to directly test these hypotheses later in this dissertation.

Alkali-labile damage

The results shown in *figure* 11 also bear on the generation of alkali labile damage by bleomycin. The diminuation of intensity of the full length bands, and perhaps of the first cleavage band (at 5'C-G-A3'), after alkali treatment indicate alkali labile damage was produced by bleomycin. No new sites were identified after the alkali treatment, indicating that the locations of the alkali labile damage were the same as those of the previously described cleavage sites. An

interesting observation was made for the strong bands just below 5'T-G-A3' and 5'A-G-C3' of sequence III (*figure* 11). The bands occur intermediate to that expected for cleavage at either the expected base or the subsequent base. This indicates the bands are due to the occurrence of both cleavage and alkali labile damage on the same molecule. The alkali labile damage must obviously be on the 3' side of the cleavage site to be visible when the label is at the 3' terminus of the oligonucleotide. The occurrence of molecules containing both alkali labile damage and cleavage while a large fraction of the oligonucleotide molecules remained uncut suggested that cleavage and alkali labile damage could be occurring concurrently. An alternative explaination is that the events were separate but cooperative, i.e. one promoted the subsequent occurrence of the other.

There were no sites indicating both cleavage and alkali labile damage for 3' end labelled sequence I. When this sequence was labelled at the 5' end, however, cleavage and alkali-labile damage occurring in the same molecule was seen (indicated by the arrow in *figure* 12). Note that the structure of the bleomycin generated end is different from that of the Maxam and Gilbert chemical cleavage reactions making identification of the 5' end labelled oligonucleotide fragments more difficult than those of the 3' end labelled oligonucleotides used in the previous experiment.The locations of the damage sites are estimated in the diagram below.



The alkali sensitive fragment producing the band identified by the arrow appears to be due to cleavage at the central G-C and alkali labile damage at a site closer to the 5' terminus. The fact that the closest G-A sequence gains intensity after the alkali treatment suggests that this is the site of the alkali labile damage.

The following experiment was designed to test the hypothesis that the alkali labile damage occurred concurrently with the cleavage reaction. Sequence III labelled at the 3' end was used with Fe(II)-bleomycin at a drug to oligonucleotide ratio of 36. Normal reaction conditions of Na-phosphate buffer (pH 7.6) and 10 mM dithiothreitol were used, however, competing unlabelled DNA was added at three different levels. Each of the three reaction mixtures was allowed to react for 1 minute and 10 minutes. If the cleavage and alkali labile damage occur concurrently as an initial reaction with an intact oligonucleotide molecule, the band produced should increase with time similar to the bands representing only cleavage sites. Likewise, the competing DNA should decrease the intensity of the band representing the putative concurrent damage site to the same degree as that of the simple cleavage sites. The results can be seen in *figure* 13. Although the bands are faint, it can be seen that the band representing the putative concurrent damage site (marked by an

arrow in *figure* 13) only becomes distinct after the longer reaction time. Secondly, increasing the amount of competing DNA decreased the intensity of the band representing the putative concurrent damage site to a greater extent than that of the bands marking simple cleavage sites. We interpret these results to mean that the alkali labile damage occurred separately and subsequent to the cleavage reactions.

Figure ° 13

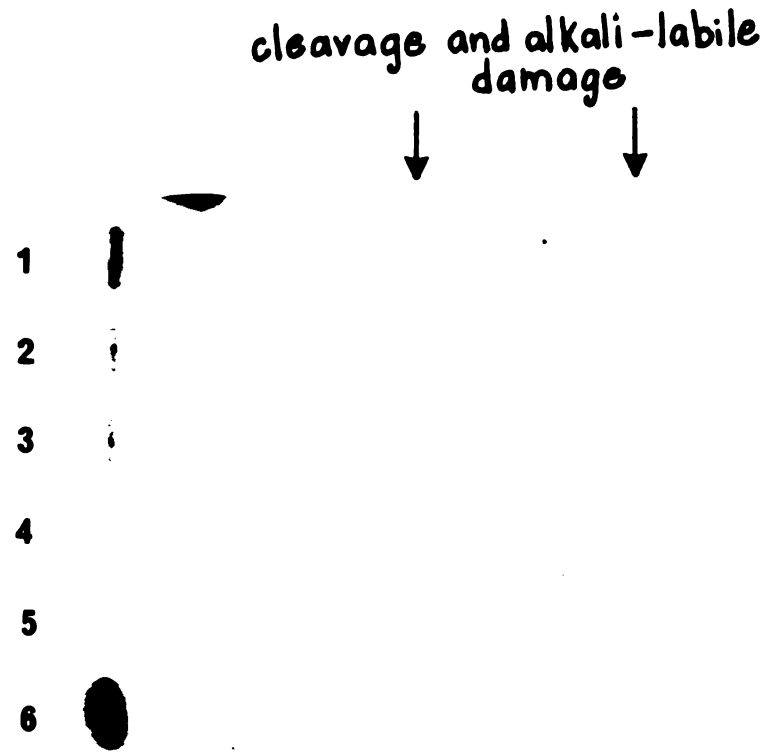


Figure 13. Cleavage and alkali labile damage of ~0.1 uM 3' (^{32}P)ddA labelled dodecamer as a function of the concentration of unlabelled competing DNA and length of reaction. Bleomycin-Fe(II) concentration was 3.6 x 10^{-6} M. Unlabelled, sonicated calf thymus DNA was added as indicated. The reaction was carried out in 20 mM Naphosphate, 10 mM DTT buffer on ice in a 10 ul reaction volume. An aliquot from each reaction was removed at 1 minute and quenched with excess EDTA, the remainder was quenched at 10 minutes. Samples were assayed by analytical 20% PAGE. Location of bands was made by comparison to previous gels of the same sequence (for example see *figure* 11).added at 0.025 ug, 0.1 ug, or 1.0 ug to the 10 ul total volume.
1) Control, 10' incubation with Fe(II) present, no bleomycin.
2) 1' bleomycin reaction in the presence of 0.025 ug competing DNA.
3) 10' bleomycin reaction in the presence of 0.025 ug competing DNA.
4) 1' bleomycin reaction in the presence of 0.1 ug competing DNA.
5) 10' bleomycin reaction in the presence of 0.1 ug competing DNA.
6) 1' bleomycin reaction in the presence of 1.0 ug competing DNA.

The use of synthetic, hairpin DNA to model bleomycin cleavage opposite a single strand break.

The indication that cleavage on one strand by bleomycin could promote cleavage on the opposite strand encouraged us to design experimental systems to test aspects of cross strand cleavage directly. We designed and modified the oligonucleotide, TK01, as described in *Methods*. Both ends of this 53 base oligonucleotide fold back to form intramolecular base-pairs yielding two hairpin structures. The sequence was designed to include several preferred bleomycin cleavage sites. The fact that the duplex structure is actually a single DNA strand allowed us to assay directly cleavage sites on both sides of the duplex. The 3' and 5' termini of the double hairpin oligonucleotide fold back and meet each other with a single base gap between them. This was designed to model the gap generated by a bleomycin generated single strand break. The termini themselves were then modified to investigate the detailed effects of the structure and charge of the nick on subsequent cleavage opposite the modified site.

The three nick models reported here are described in the following diagram. The ends were modified as indicated by the bold print.

1) ...**G3'-p-ddA ho-5'C**...
2) ...**G3'OH__=p5'C**...
3) ...**G3'-p=__HO-C**...

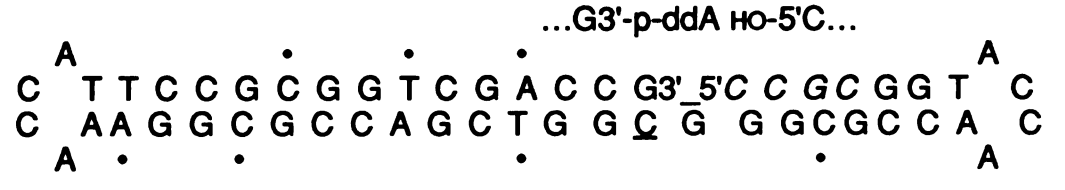
```
  A                                                   A
C  T T C C G C G G T C G A C C G3'_5'C C G C G G T  C
C  AA G G C G C C A G C T G G C  G  G G C G C C A  C
  A                                                   A
```

Before performing the experiments reported here, we determined that the oligonucleotide did form a duplex structure with the correct base pairing by digesting it with a restriction enzyme whose recognition site is present in the sequence. The enzyme cleaved the oligonucleotide at 25°. Our experiments with bleomycin were performed at 0°, so we were confident that the proper base pairing occurred.

The following diagrams depict the results of cleavage of the oligonucleotide with three different modifications of the termini. Major cleavage by bleomycin is marked by the symbol "•". Less distinct cleavage is indicated by the symbol " | ". The sequence that was unreadable in the sequencing gel used to assay the particular experiment is designated in *italics*. The modifications performed at the termini are depicted above each diagram.

1. Control: gap is filled without an increase in charge.

The 3' terminus was labelled with [å-32P]Dideoxyadenosine triphosphate and Terminal Transferase,[6] the 5' terminus was left a hydroxyl. Note that the gap has been filled, but there is no additional charge relative to normal DNA. See *Figure* 14 for the details of the reaction conditions.



[6] see appendix entry for 3' labelling protocols.

Significant cleavage occurred at the expected cleavage sites; however, there was a conspicuous absence of cleavage across from the filled in nick, identified by C. This is consistant with our hypothesis for cross strand cleavage. Bleomycin reacts with unmodified DNA only with base-paired sequences. The mismatch of dideoxyadenosine opposite a guanine would allow only a loose base-pairing, no stronger than a terminal A:T base-pair, thus easily disrupted.[7] The pertinent G:C base-pair adjacent to the nick is expected to be intact. However, apparently this 3' terminal modification prevented the normal mode of cleavage at this preferred site. The results of bleomycin cleavage of the entire sequence are given in *figure* 14. A closeup focusing on cleavage just opposite the nick model is given in the composite *figure* 17.

[7] Model building shows two hydrogen bonds can form for the A:G pair. In solution, however, these bases do not base pair: see
a) Biophysical Chemistry, Part I, C.R Cantor and P.R. Schimmel (eds.), W.H. Freeman and Co., San Francisco, pp.322-325; and
b) Lord, R. C., G.J. Thomas Jr. (1968), *Devel. Appl. Spectroscopy* **6**:179.

Figure ° 14

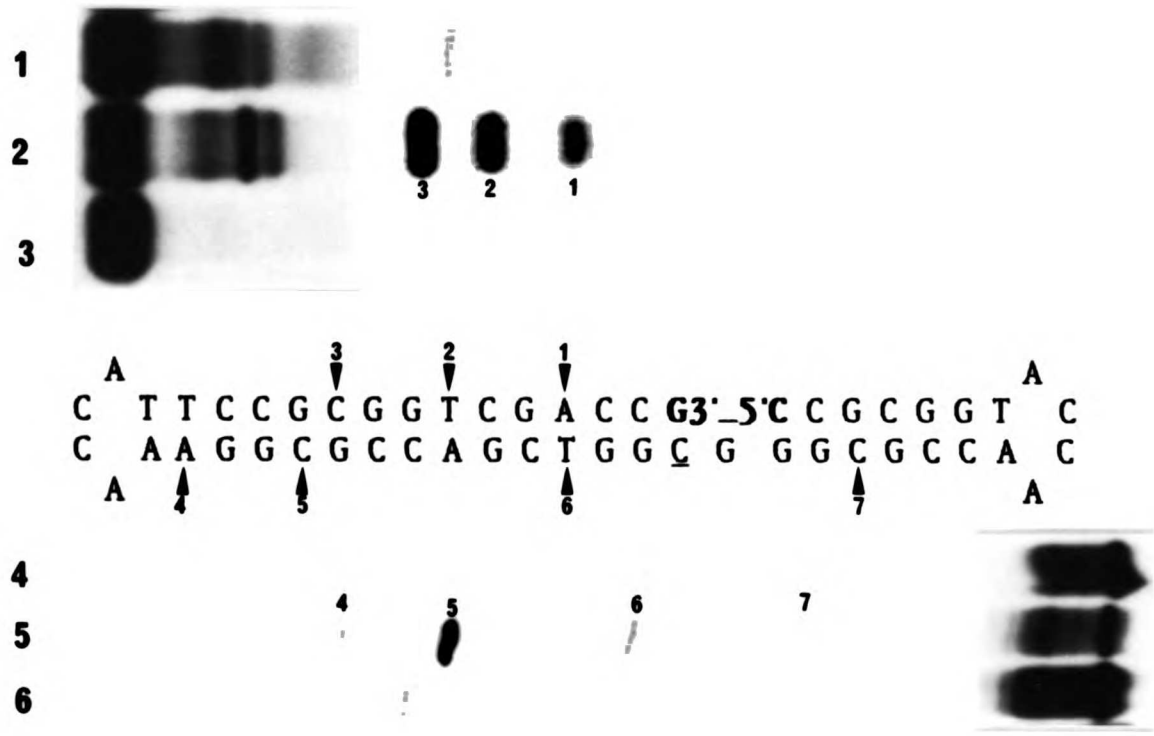


Figure 14. Double-hairpin nick model: control. Bleomycin cleavage of the double hairpin oligonucleotide with the gap filled without an increase in charge: ...G_{dd}A HOC.... The labelled oligonucleotide was incubated with 3.4 uM Fe(II)-bleomycin for 1' on ice. The buffer contained 25 mM NaCl, 10 mM Na-phosphate (pH 7.6), 5 mM dithiothreitol and 1 ug unlabelled sonicated calf thymus DNA. These conditions limit the reaction to initial cleavage sites. Samples were analyzed by analytical PAGE under denaturing conditions. Chemical sequencing of the oligonucleotide was performed and run alongside to identify bleomycin cleavage locations.

1) G sequence markers
2) Reaction with Fe(II)-bleomycin
3) Control, Fe(II), no bleomycin
4) Control, Fe(II), no bleomycin
5) Reaction with Fe(II)bleomycin
6) G sequence markers

2. Increase charge at the 5' terminus, the gap is maintained.

In this experiment, the 3' terminus was not modified, but the 5' terminus was phosphorylated using [å32P]ATP and Polynucleotide Kinase[8]. This modification produces an increase in charge, relative to native DNA, on the 5' side of the gap. This modification only partially mimics a bleomycin generated single strand break (vide supra).

Bleomycin cleaves at the normal cleavage sites, similar to the pattern shown above for the 3'-pddA labelled control. However, an additional major site now occurs opposite the phosphorylated nick (underlined). Also of note is the fact that relatively more cleavage was detected along the strand with the terminal phosphate. This model maintains the gap and has half of the increase in charge that would occur at a bleomycin generated single strand break. *Figure* 15 shows the results for cleavage of the entire oligonucleotide. We focus on cleavage opposite the nick model in *Figure* 17.

[8] see appendix for the 5' end labelling protocol.

Figure ' 15

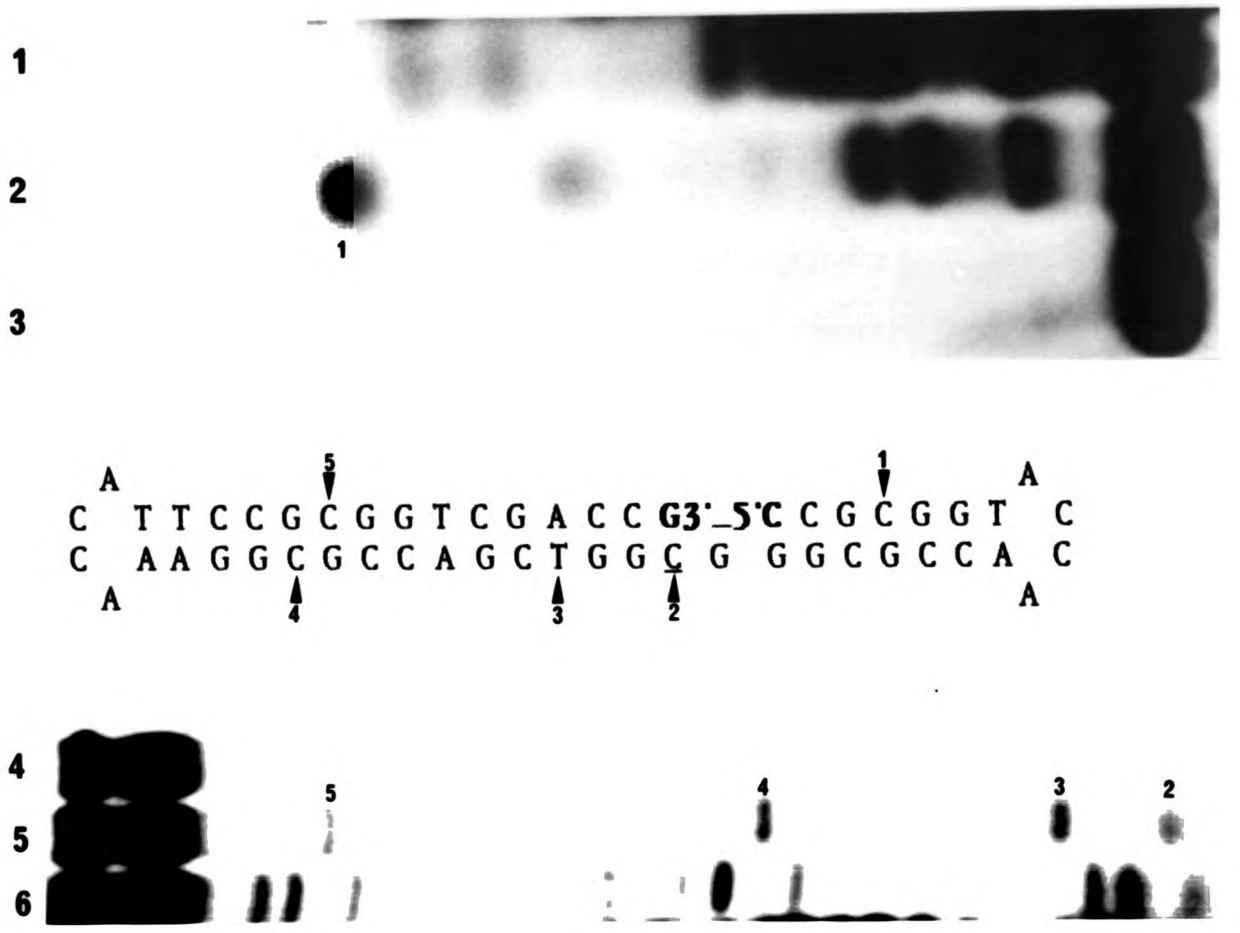
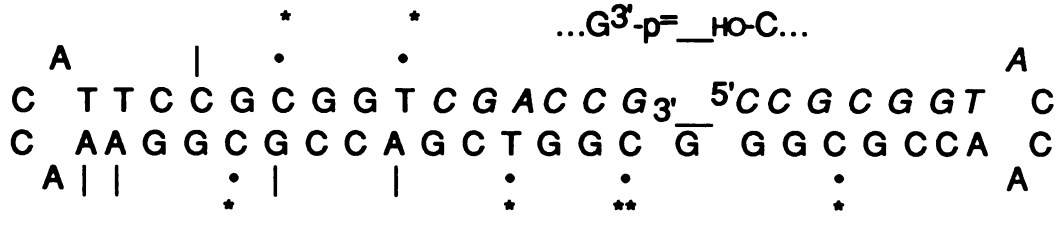


Figure 15. Double-hairpin nick model: Increased charge at the 5' terminus. Bleomycin cleavage of the double hairpin oligonucleotide with the gap maintained and a 5' terminal phosphate: ...GOH "pC.... The labelled oligonucleotide was incubated with 3.4 uM Fe(II)-bleomycin for 1' on ice. The buffer contained 25 mM NaCl, 10 mM Na-phosphate (pH 7.6), 5 mM dithiothreitol and 1 ug unlabelled sonicated calf thymus DNA. These conditions limit the reaction to initial cleavage sites. Samples were analyzed by analytical PAGE under denaturing conditions. Chemical sequencing of the oligonucleotide was performed and run alongside to identify bleomycin cleavage locations.

1) G sequence markers
2) Reaction with Fe(II)-bleomycin
3) Control, Fe(II), no bleomycin
4) Control, Fe(II), no bleomycin
5) Reaction with Fe(II)bleomycin
6) G sequence markers

3. Increased charge at the 3' terminus, the gap is maintained. For this model, a series of reactions were performed to generate a phosphorylated 3' terminus, the 5' end was left an unmodified hydroxyl. We used this model as a substrate for bleomycin in the presence of different levels of unlabelled, competing DNA. We used the competing DNA in an attempt to limit the amount of cleavage by the drug. In that way we hoped to determine the location of the initial site of cleavage by the drug. In *Figure* 16 results of cleavage of the 3' phosphorylated oligonucleotide are shown as a function of competing, unlabelled CT DNA. The following diagram summarizes the result of this experiment.



* Intensity of band increases with lower amounts of competing DNA.
** Intensity of band increases with larger amounts of competing DNA.

Cleavage is seen at all the preferred sites resolved on the gel, as expected. The modification, maintaining the gap while increasing the negative charge at the terminus promotes cleavage opposite the nick model. This is similar to the results of the previous experiment. Comparison of the amount of cleavage as a function of competing DNA was difficult to assess, cleavage did still occur at all the sites in the presence of high amounts of competing DNA. However, comparison of lanes 4 and 5 does indicate some difference between cleavage opposite the 3' phosphorylated nick and cleavage at the adjacent "normal" cleavage sites as a function of competing DNA. The bands for the two native cleavage sites (a and c) in *figure* 17, are more intense with less competing DNA. The site opposite the nick site (b), however, is less intense with less competing DNA (lane 5) than it is with more competing DNA (lane 4). This is the

result one would expect if the competing DNA competes effectively for bleomycin against the native sites on the oligonucleotide; whereas the site opposite the nick model is preferentially attacked by the drug. We suggest that higher amounts of competing DNA decreased the amount of cleavage at the normal sites on the oligonucleotide relative to the site opposite the nick where the dianionic terminal phosphate next to the gap allowed a stronger electrostatic interaction with the cationic drug.

Figure ° 16

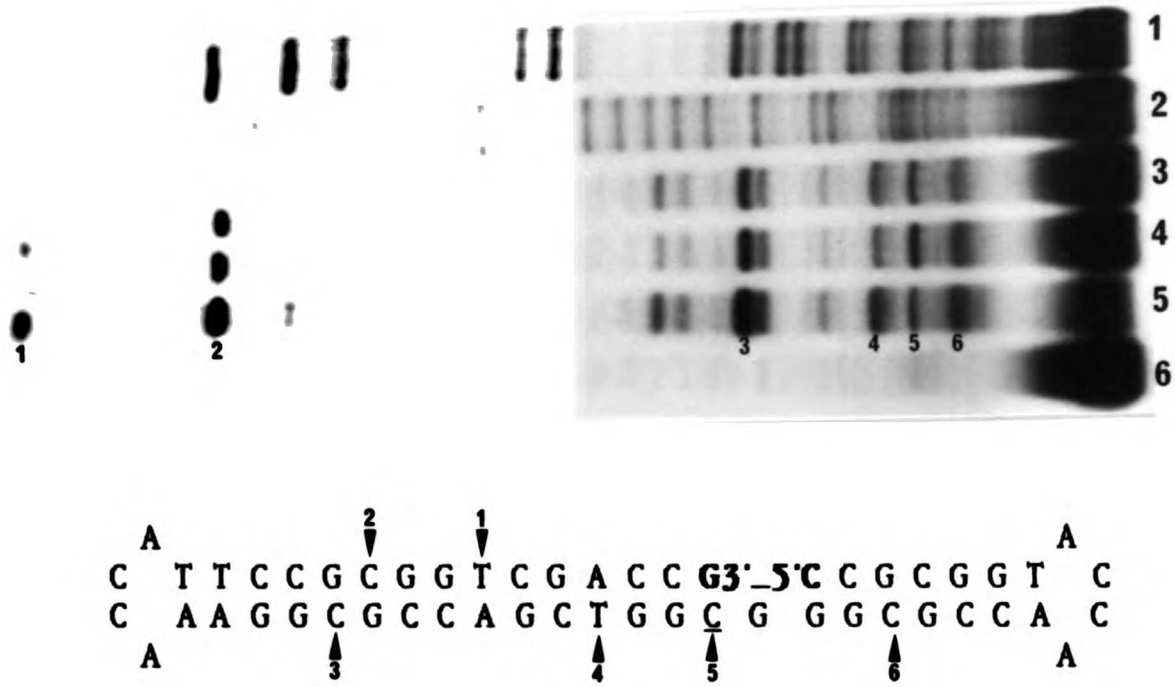


Figure 16. Double-hairpin nick model: Increased charge at the 3' terminus. Bleomycin cleavage of the double hairpin oligonucleotide with the gap maintained and a 3' terminal phosphate: ...Gp= HOC.... The labelled oligonucleotide was incubated with 3.4 uM Fe(II)-bleomycin for 1' on ice. The buffer contained 25 mM NaCl, 10 mM Na-phosphate (pH 7.6), 5 mM dithiothreitol and competing, unlabelled, sonicated, calf thymus DNA as indicated. Increasing amounts of unlabelled DNA were expected to progressively compete for normal cleavage sites, while the site opposite the charged nick was not expected to decrease linearly. Samples were analyzed by analytical PAGE under denaturing conditions. Chemical sequencing of the oligonucleotide was performed and run alongside to identify bleomycin cleavage locations.

1) C + T sequence markers
2) G + A sequence markers
3) Reaction with Fe(II)-bleomycin, 1 ug CT DNA
4) Reaction with Fe(II)-bleomycin, 3 ug CT DNA
5) Reaction with Fe(II)-bleomycin, 6 ug CT DNA
6) Control, Fe(II), no bleomycin, 1 ug CT DNA

Figure • 17

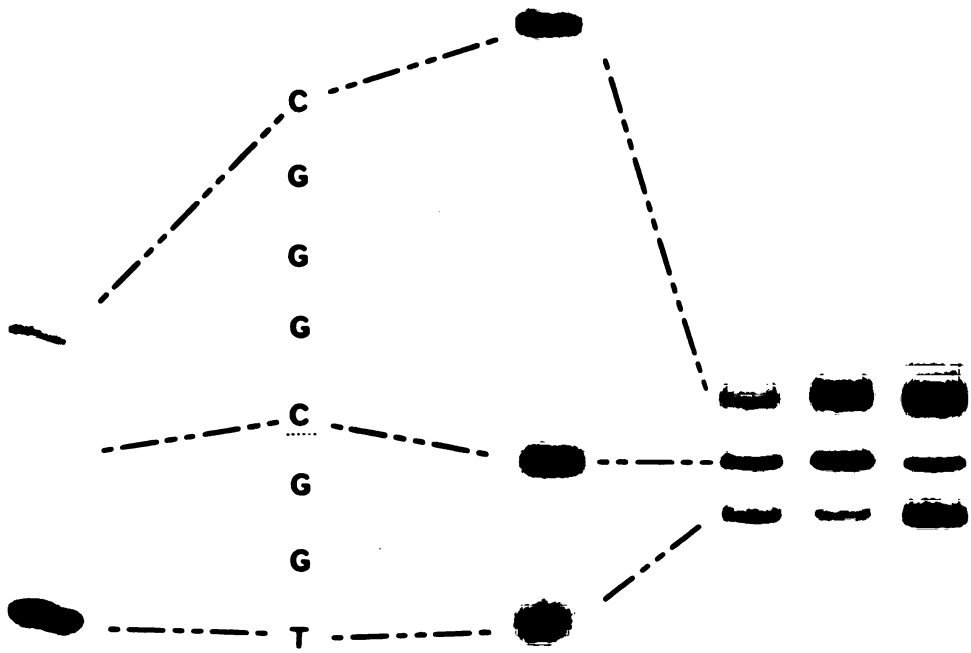


Figure 17. Cleavage opposite double hairpin nick models: composite of *figures* 14, 15, and 16. Reaction conditions are described in the appropriate figure legend. This figure focuses on bleomycin cleavage opposite the three nick models (see text). The two neighboring unmodified sites are also shown.

The hairpin DNA used in the experiments described above presented technical difficulties for its analysis by high resolution electrophoresis. The phenomenon of compression is described by Maxam and Gilbert (1980) as a result of persistent secondary structure in the DNA being sequenced. Additionally, the hairpin structure made the enzymatic modification reactions difficult. We also found the double hairpin system refractory to the production of a model where both the 3' and 5' termini were phosphorylated with a single phosphate and the gap between the termini maintained. A sort of Heisenberg principle obtained, the more data obtainable from a single system, the more uncertain each datum became. In short, this experimental system was overdesigned. We, therefore, devised a system that would test, directly and more simply, the effect of a bleomycin generated single strand break on subsequent cleavage on the opposite strand.

Models for Bleomycin cleavage opposite single strand nicks.

We designed the following experimental system to allow us to model bleomycin's relative activity at a cleavage site opposite a previously generated nick vs. a site opposite a native (uncut) strand. A series of non-palindromic oligonucleotides were prepared: a single template quadecamer (14-mer), a heptamer complementary to the 7 bases of the 3' end of the template, a hexamer complementary to the 6 bases of the template's 5' end, and a quadecamer complementary to the entire template sequence.[9] The two shorter oligonucleotides were modified as described in *Methods* to allow the preparation of two different nick models. With the appropriate oligonucleotides in hand, they were combined stoichiometrically with the template

[9] Synthesis by J. Barnett on an ABI automated synthesizer. Purification was done by TJK as described in *Methods*.

oligonucleotide, under conditions promoting stable base-pair formation, to form three different duplex structures. A portion of the template oligonucleotide was labelled at the 3' end with [^{32}p]-dideoxyadenosine to allow assay of the final reaction products by autoradiography. One nick model closely mimicks the structure of a bleomycin generated single strand break: a single base gap on one strand is bordered by dianionic phosphate groups on the opposing 3' and 5' termini (...Gp= =pC...). The second nick model is composed of a single base gap bordered by uncharged hydroxyl groups on the adjacent termini (GOH HOC). Comparison of these two models allows us to assess the relative importance of the gap and the negatively charged termini generated by a bleomycin reaction on subsequent activity by the drug on the opposite strand. The third model was composed of the complementary quadecamer and the template oligonucleotide to form the intact native strand opposite the site of interest (...G-A-C...). In addition to the site pertaining to the nick models, there are two other preferred bleomycin nick sites on the template strand present in all three models. These are at internal positions where normal duplex structure exists under the conditions used for the experiments. The three models are shown in the diagram below. The labelled template strand is below the nick site strand, the cleavage sites are underlined.

I. Native:
5'C C A C G G G A C G G A C G3'
3'ddA G G I G C C C I G C C I G C5'

II. Charged nick:
5'C C A C G G Gp= =pC G G A C G3'
3'ddA G G I G C C C I G C C I G C5'

III. Uncharged nick:
5'C C A C G G GOH HOC G G A C G3'
3'ddA G G I G C C C I G C C I G C5'

Each of the three models was separately incubated with bleomycin under identical conditions (see *Methods*). Note that the Fe(II)-bleomycin concentration was equal to that of the oligonucleotide substrate. The autoradiographs demonstrating the sites cleaved by bleomycin is pictured in *Figures* 18 and 19.

Figure ' 18

Nick Models 1) Native; 2) Charged nick: $G_{p=}$ =pC; 3) Uncharged nick: G_{OH} HOC

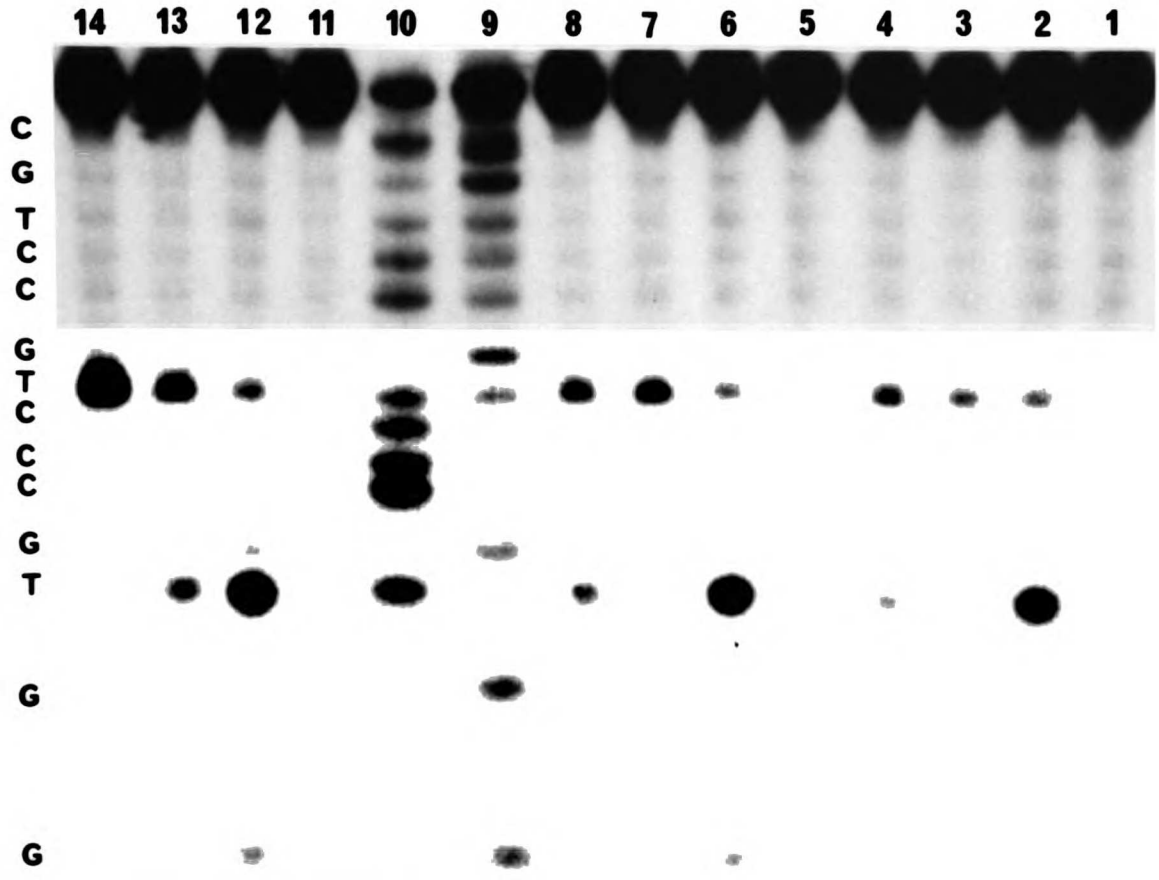


Figure 18. Bleomycin cleavage of nick models (see text for complete structure). Fe(II)-Bleomycin (BLM) was incubated with each of the three model systems on ice for 1' or 10' in a buffer containing 380 mM NACl, 10 mM Na-phosphate (pH 7.6), and 5 mM dithiothreitiol. Bleomycin/oligonucleotide ratio was 1, at a concentration of 4.8 uM. Sequence markers were generated by the methods of Maxam and Gilbert (1980). Samples were assayed by analytical PAGE and autoradiography.

1 minute reaction time:

1) Control, Fe(II) but no BLM
2) BLM reaction with I. native structure
3) BLM reaction with II. charged nick
4) BLM reaction with III. uncharged nick

10 minute reaction time:

5) Control, Fe(II) but no BLM
6) BLM reaction with I. native structure
7) BLM reaction with II. charged nick
8) BLM reaction with III. uncharged nick
9) A + G sequence markers
10) C + T sequence markers

30 minute reaction time:

11) Control, Fe(II) but no BLM
12) BLM reaction with I. native structure
13) BLM reaction with II. charged nick
14) BLM reaction with III uncharged nick

Figure ' 19

Nick Models 1) Native; 2) Charged nick: $G_{p=}$ =pC; 3) Uncharged nick: G_{OH} HOC

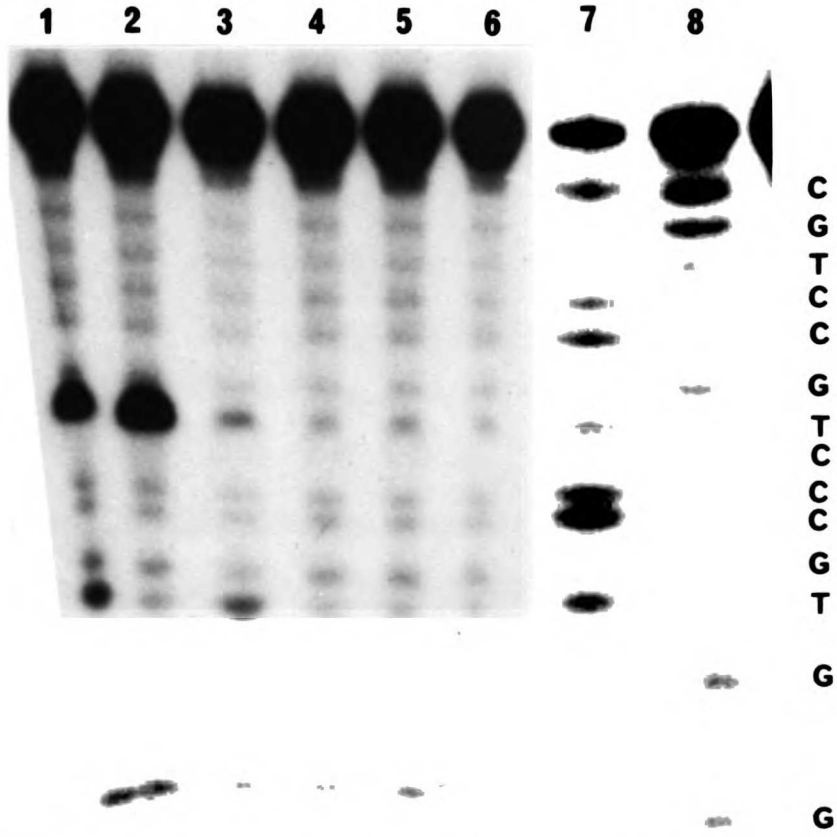
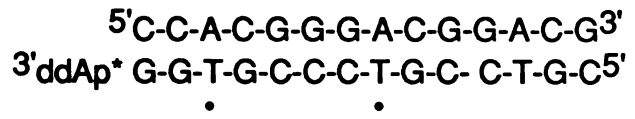


Figure 19. Bleomycin cleavage of nick models (see text for complete structure). Fe(II)-Bleomycin (BLM) was incubated with each of the three model systems on ice for 30' in a buffer containing 380 mM NACl, 10 mM Na-phosphate (pH 7.6), and 5 mM dithiothreitiol. Bleomycin/oligonucleotide ratio was 1, at a concentration of 4.8 uM. Controls were run by replacing bleomycin with distilled water. Sequence markers were generated by the methods of Maxam and Gilbert (1980). Samples were assayed by analytical PAGE and autoradiography.

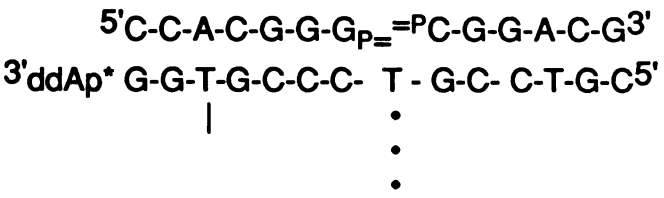
1) BLM reaction with III. uncharged nick
2) BLM reaction with II. charged nick
3) BLM reaction with I. native duplex
4) Control, III with Fe(II) but no BLM
5) Control, II with Fe(II) but no BLM
6) Control, I with Fe(II) but no BLM
7) C + T sequence markers
8) G sequence markers

The results are summarized below; modest cleavage is designated by "|", substantial cleavage is marked by "•".

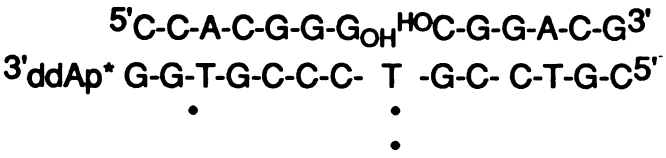
I. Native duplex:



II. Charged nick:



III. Uncharged nick:



The reaction conditions were such that no more than a single cleavage reaction was likely to occur on each oligonucleotide molecule. Comparing cleavage at the central G-I for the three models, the results are unambiguous. The activity of bleomycin opposite the uncharged nick model was significantly greater than opposite the intact, native strand. And cleavage opposite the phosphorylated nick model was dramaticly increased relative to the control, unnicked strand.

The results for cleavage at the central G-I site relative to the distal G-I site are equally clear. For the control model with the intact strand, there was a slight preference for the G-I site closest to the 3' labelled end relative to the central G-I although both sites were attacked by the drug. In comparison, cleavage of the uncharged nick model oligonucleotide was reversed. There was an increase in cleavage at the central G-I, opposite the nick, relative to the G-I near the 3' end. Finally, the phosphorylated nick model directed all bleomycin activity to the

G-I opposite the charged nick. There was essentially no cleavage at the G-I near the 3' end.[10]

This experiment demonstrates that cleavage occurs preferentially opposite a nick, even when it isuncharged. However, there is a tremendous enhancement of cleavage opposite the nick when it is phosphorylated. Since the structure of this latter model, the phosphorylated nick, is very similar to that of an authentic bleomycin generated nick, the results for cleavage opposite a single strand break generated by the drug are expected to be the same.

[10] There was no cleavage of any of the models at the G-I site closest to the 5' end. We can only speculate as to the reason for this. One possibilty is that the salt concentration used for the experiment was sufficiently high to promote a B—>Z transition at the 5' terminal (C-G...) dinucleotide, thereby disrupting the bleomycin cleavage site at this position. The fact that the control intact model is cleaved at the other two sites indicates that there was not a problem with duplex formation.

CONCLUSIONS

Enhanced cleavage opposite single strand breaks. The importance of electrostatic interactions for bleomycin binding to DNA was discussed in the *Introduction*. Our results demonstrated that the amount and specificity of double strand cleavage by the drug is a function of ionic strength. The experiments using palindromic oligonucleotides indicated that there could be a cleavage reaction occurring across from a preferred single strand cleavage site subsequent to nicking by the drug at the latter site. We began to study cross-strand cleavage directly with hairpin oligonucleotides. Modification of the termini of these oligonucleotides allowed us to demonstrate the affect of the structure and charge of a nick on cleavage of the opposite strand by bleomycin.

From these results, we developed the hypothesis that bleomycin generates non-random double strand breaks in the following way. Initial single strand breaks are produced at the preferred sequences. The reaction produces a 3'-phosphoglycolate and an unmodified 5' phosphate (dianionic monoesters) with a gap of one nucleotide in between. The negative charge at the damaged site is twice that of intact native DNA. Electrostatic interaction of the cationic Fe(II)-bleomycin with the damaged site is therefore enhanced. This promotes cleavage of the opposite strand to directly produce a double strand cleavage. The experiments performed for *figures* 18 and 19 tested the hypothesis. The results strongly support the hypothesis and suggest this is the major mechanism for non-random double strand cleavage by bleomycin.

METHODS

Restriction enzyme mapping.

Materials.

Restriction enzymes were purchased from either Bethesda Research Labs, New England Biolabs, or Pharmacia P-L Biochemicals. Bleomycin-A_2 was a generous gift of S. Hecht. Normal and low gelling temperature agarose was purchased from FMC, Marine Colloids, Inc.

The supercoiled plasmid, pBR322, was grown to a high copy number in HB101 (recA-) E. Coli using standard methods.[1] These included minimal growth media supplemented with uridine and chloramphenicol amplification. A cleared lysate was prepared from lysozyme and Triton X-100 lysed cells. The covalently closed circular DNA was purified by CsCl equilibrium density (isopycnic) centrifugation.

Cleavage of plasmid DNA by bleomycin.

Purified form I or form III, plasmid DNA was incubated with Fe(II)-bleomycin-A_2 in Tris buffer (pH 7.5) containing either ß-mercaptoethanol or dithiothreitol as reductant. Further details of experimental procedures are given in the figure legends.

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[1] a. Kahn, M., Kolter, R., Thomas, C., Figurski, D., Meyer, R., Remaut, E., and Hellinski, D.R. (1980) *Methods in Enzymology* **68:** 268-280.
b. Norgard, M.V., Emigholz, K., and Monahan, J.J. (1979) *J. Bact.* **138:** 270-272.

Preparation of bleomycin generated form III plasmid DNA for restriction enzyme mapping.

Approximately 115 picomoles of pBR322 (>95% supercoiled) was incubated with 2.3 nanomoles Fe(II)-bleomycin (Fe(II)-blm/pBR322 = 20) in10 mM Tris (pH 7.8) and25 mM ß-mercaptoethanol in a total volume of 1.5 ml for 10 minutes at 37°. The reaction was quenched with the addition of EDTA to 25 mM and SDS to 0.1%. Bleomycin was removed by passage over a Dowex AG 50W-X8 (100-200 mesh) column equilibrated with NaAcetate buffer, pH 5.4. The eluted DNA was ethanol precipitated. The full length (+/- 40 base pairs) linearized pBR322 generated from the bleomycin reaction was separated from the uncut and nicked pBR322 by preparative gel electrophoresis with low melting temperature agarose.[2]

Radiolabelling for restriction mapping.

Two methods of labelling for restriction enzyme mapping were used. In the first, the bleomycin generated 5' ends were radiolabelled via [gamma ^{32}P]-ATP and T4 PNK after removal of the unlabelled 5' phosphates by alkaline phosphatase reaction (Maniatis et al., 1982). The labelled DNA was then digested with one or more restriction enzymes, as described below.

The second method was to perform the restriction enzyme digests of the bleomycin linearized plasmid prior to labelling. All enzymes used produce underhanging 3' ends (See Maniatis et al., 1982). These can be labelled by "filling in" using [alpha ^{32}P]-NTPs and T4 DNA polymerase (Maniatis et al., 1982). The bleomycin generated ends are not labelled by this enzyme without prior digestion with a 3' —> 5' exonuclease (Niwa and Moses, 1981).

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[2] Wieslander, L. (1979) *Analyt. Bioch.* **98:** 305-309.

Restriction enzyme mapping.

The location of double strand breaks can be estimated by the technique of restriction enzyme mapping.[3] See Appendix for a description of this technique as applied to DNA cleaving drugs. Incubation of the DNA with the enzyme was carried out as suggested by the manufacturer. The amount of DNA used was for each set of reactions was adjusted according to the specific activity achieved by the labelling reaction. The amount of enzyme used was adjusted accordingly. Analysis of the double digest products was by neutral agarose gel electrophoresis.

Cleavage of oligonucleotides having specified sequences

Palindromic oligonucleotides. Several synthetic, palindromic oligonucleotides were designed to study the effect of neighboring sequences on cleavage by bleomycin. These were synthesized by C.L. using phosphoramidite chemistry on an ABI synthesizer.[4]

(1) NO01

5'–C–G–A–G–A–G–C–T–C–T–C–G
G–C–T–C–T–C–G–A–G–A–G–C–5'

(2) NO02

5'- G C C T G T A C A G G C
C G G A C A T C T C C G -5'

(3) GH1

5'- A T G A A G C T T C A T
T A C T T C G A A G T A -5'

The oligonucleotides were radiolabelled at the 3' end using [alpha ^{32}P]dideoxyATP and Terminal Deoxynucleotidyl Transferase. Sequence

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[3] a. Parker, R.C. (1980) *Methods in Enzymology* **65**: 415-429.
b. Danna, K.J. (1980) *Methods in Enzymology* **65**: 449-468.

[4] C.L. is Corey Levenson, technical assistance was by Jennifer Barnett, at Cetus Corp.

markers were generated using the chemical sequencing methods of Maxam and Gilbert (1980). The labelled oligonucleotides were incubated with Fe(II)-bleomycin in Na-phosphate (pH7.8) buffer. Further details of the reaction are given in the firgure legends.

Bleomycin cleavage reaction conditions.

Unless otherwise stated, reactions were run on ice in 10 mM Na-phosphate buffer containing 5 mM Dithiothreitol. Fe(II) was made immediately prior to addition to the reaction mixture from $FeSO_4$-$7H_2O$.

Double-hairpin nick models.

Cleavage by bleomycin at the sequence ...ACCGCCCG... would produce an internal single strand nick ...ACCGx= =pCCG... where x= stands for the phosphoglycolate generated by the drug (vide supra). We wished to test the hypothesis that the increased negative charge produced by the bleomycin reaction could direct cleavage by the drug to the opposite strand. A 53 base oligonucleotide was designed to form two hairpin loops bringing the 3' and 5 ends towards one another with a single nucleotide gap between them. Various modifications of the termini were performed to create a structure similar to that of a bleomycin generated nick. The effect of these modifications on subsequent cleavage of the opposite strand by bleomycin was determined. It should be noted that use of the hairpin structure allows investigation of both strands of the duplex simultaneously.

The following diagram shows the oligonucleotide folded back upon itself to form the double hairpin with a single base gap. The modifications performed are also shown.

TK01

A A

C T T C C G C G G T C G A C C G 3' 5' C C G C G G T C
C A A G G C G C C A G C T G G C G G G C G C C A C
A A

1) ...GpddA HOC...
2) ...GOH __ =PC...
3) ...GP=__ HOC...

Synthesis of a double-hairpin nick models.

1) ...GpddA HOC...

A control, with the gap filled by the mismatch ddA, with ^{32}P internal, and no external 3'-phosphate. This oligonucleotide is produced by reaction of dideoxyadenosine triphosphate and terminal deoxynucleotidyl transferase, (TdT), with TK01.[1]

2) ...GOH __ PC...

5' end labelling was accomplished with ATP and Polynucleotide Kinase (PNK) using the forward reaction for blunt or recessed ends.[5] Increased temperature, 90°, was used for the initial melting step.

3) ...GP _ HOC...

Scheme 1. The oligonucleotide was tailed at the 3' end with [å-32P]rCTP or unlabelled ribo-CTP and Terminal Deoxynucleotide Transferase (TdT) to produce ...G(prC)$_n$ HO-C..., with n=1 or 2 generally.[6] Pancreatic Ribonuclease A was used to cleave the 3'-tail to leave the single 3', 5'-diphospo-riboC at the

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[5] Maniatis T., E.F. Fritsch, and J. Sambrook, Molecular Cloning, A Laboratory Manual, p.124, Cold Spring Harbor, 1982.

[6] a. R. Roychoudhury and R. Wu, *Methods in Enzymology* **65**: 43-62 (1980).

3' terminus with the 5' terminal hydroxyl unchanged.[7] If n was 1, the product of the reaction was ...GprCOH HOC....

Scheme 2. Alternatively, ...GprCP HOC.... is formed directly using 3', 5' diphopho-cytidine and T4 RNA ligase.[8,7]

...GprCOH HO-C...

Removal of the 3'-terminal phosphate is accomplished with E. Coli alkaline phosphatase (BAP). Bovine intestinal alkaline phosphatase (CIP) may also be used.[9] A single precipitation is used to purify the oligonucleotide for subsequent periodate/alkali cleavage (vide infra).

...GP _ HOC...

An adaptation of published periodate/alkali cleavage methods was used to generate the oligonucleotide with a single phosphate as the 3'-terminus.[3a,10] The oligonucleotide, TK01.II, was dissolved in 100ul NaAc(0.15M, pH adjusted to 5.3 w/ HOAc); carrier tRNA (1ug) was added. NaPeriodate was added to this (~100 fold excess compared to ~45pmoles carrier tRNA, assuming [oligo] <<

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[7] Pancreatic ribonuclease A is specific for(...Py-p-X...) , with cleavage between p-X, leaving the 3'-p.

[8] G. Krupp and H.J. Gross "Sequence analysis of in vitro ^{32}P-labelled RNA" in THE MODIFIED NUCLEOSIDES OF TRANSFER RNA II, pp11-58. P.F. Agris (ed.) New York, '83.

[9] a. H. Fraenkel-Conrat and A. Steinschneider, *Methods in Enzymology* **12B**:243-246 (1967).

b. G. Krupp and H.J. Gross, "Sequence Analysis of In Vitro ^{32}P-Labelled RNA", in The Modified Nucleosides of Transfer RNA II, P.F. Agris and R.A. Kopper (eds.), pp11-58, 1983 Alan R. Liss, Inc., New York.

c. B. West lab procedure, personal communication, used 3/20/85

[10] Lee, J.C., H.L. Weith, P.T. Gilham, Biochem 9(1): 113-118 (1970) "Isolation and characterization of terminal polynucleotides fragments from ribonucleic acids."

[tRNA]). The reaction mixture was incubated on ice, 30', in the dark. The products of this reaction were cleaned up by ethanol precipitation. The pelleted oligonucleotides are then dissolved with 100ul 1M piperidine and incubated at 95° for 30' in sealed tubes. Piperidine was removed by repeated lyophilization.

Purification of the modified oligonucleotides was accomplished by preparative electrophoresis at 50°C on a 20% polyacrylamide gel with 7.5M urea, diffusion of the oligonucleotides from the cut out bands, removal of acrylamide and other contaminants by NACS-37™ or Sep-pak™ mini column chromatography, and finally ethanol precipitation. The products of the bleomycin reactions were assayed by analytical denaturing PAGE and compared to the chemical sequencing reaction products of the same oligonucleotide.[11]

Non-hairpin nick models

The following three duplex sequences were designed to directly test the hypothesis that that bleomycin preferentially cleaves opposite bleomycin generated nicks. Four oligonucleotides were designed, synthesized, and purified as components for the model systems. Synthesis was by phosphoramidate chemistry, performed by Jennifer Barnett on an ABI synthesizer.[12] These were enzymatically and/or chemically modified, and purified as needed.

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[11] Maxam and Gilbert ref, w/ modifications for oligos.

[12] The synthesis of oligonucleotides at nominal cost is a service generously provided by W. Rutter of U.C.S.F.

The following diagram shows the duplex structures of the three nick models used in our experiments.

I. Native:
$^{5'}$C C A C G G G A C G G A C G$^{3'}$
$^{3'}$ddA G G T G C C C T G C C T G C$^{5'}$

II. Charged nick:
$^{5'}$C C A C G G G$_{P=}$ $=_{P}$C G G A C G$^{3'}$
$^{3'}$ddA G G T G C C C T G C C T G C$^{5'}$

III. Uncharged nick:
$^{5'}$C C A C G G G$_{OH}$ HoC G G A C G$^{3'}$
$^{3'}$ddA G G T G C C C T G C C T G C$^{5'}$

All four strands were purified by FPLC. The Pharmacia Polyanion S/I (anion exchange) column was used with a gradient of .33% B per minute. Buffer A was .05 M K-phosphate, 30% MeOH. Buffer B was the same as A with the addition of 1 M NH_4SO_4. Purified samples were desalted by standard methods and lyophilized for use.

Strand 1, $^{5'}$CGTCCGTCCCGTGG$^{3'}$ was radiolabelled using [alpha ^{32}P]-dideoxyATP and Terminal Deoxynucleotidyl Transferase (TDT). Some degradation of the oligonucleotide occured so the labelled strand was purified by preparative PAGE.

Strand 2, $^{5'}$CCACGGGACGGACG$^{3'}$ was not modified further.

Strand 3, $^{5'}$CCACGGG$^{3'}$ was used without further modification for the uncharged nick model. The strand was phosphorylated at the 3' terminus for the charged nick model. This was accomplished by tailing the 3' end with p-(riboC) using unlabelled, (ribo)CTP and TDT. The oligonucleotide with a single p(riboC) was purified by FPLC. The terminal riboC was removed to leave a

single 3'-phosphate by oxidation of the ribose with periodate and ß elimination of the diol product with piperidine.

Strand 4, $^{5'}$CGGACG$^{3'}$ was used without further modification for the uncharged nick model. For the charged nick model, the 5' terminus was phosphorylated using unlabelled, ATP and T4 PNK. Kinase is heat sensitive, so 3 fresh aliqouts of enzyme were added to the reaction mixture at half hour intervals.

Each duplex model system was formed by mixing equimolar amounts of the appropriate strands at a concentration of 4.6 uM in 21 ul volume of 380 mM NaCl. The samples were heated to 55° for a few minutes, allowed to come to room temperature, then allowed to reaneal at 4° for 1 hour before the bleomycin reaction.

The bleomycin reaction was carried out on ice in a buffer containing 380 mM NaCl, 10 mM Na-phosphate (pH 7.6), and 5 mM DTT. The reaction was initiated by the addition of Fe(II)–bleomycin, 4.8 uM, to an equimolar amount of the duplex oligonucleotide in the reaction buffer. Controls contained Fe(II) but water replaced the bleomycin. The reactions were quenched by addition of excess EDTA at various time points. The samples were lyophilized and brought up in sequencing gel sample buffer for high resolution, denaturing 20% PAGE. Chemical sequencing of 3' end labelled strand 1 was performed and run with the bleomycin cleaved samples to identify the cleavage locations. Further details of the reaction are given in the figure legends.

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APPENDIX

Restriction enzyme mapping of double strand cleavage by bleomycin

The technique of restriction enzyme mapping is well described in standard biochmistry texts (see for example: Parker, 1980; Danna, 1980).[1] The following diagram gives the basic principles for the hypothetical case of a single bleomycin cleavage site on a circular plasmid. A single double strand break will generate two fragments if subsequently digested with a restriction enzyme that cleaves the plasmid at only one position. These fragments could be derived from two possible locations of the bleomycin cleavage. A second restriction enzyme that cleaves only once, but at another postion is used to descriminate between the two sites.

[1] a. Parker, R.C. (1980) in *Methods in Enzymology* **65**: 415-428.
b. Danna, K.J. (1980) in *Methods in Enzymology* **65**: 449-467.

End labelling:

3' labelling with (p*Cp) and T4 RNA ligase[2]:

lyophilized [5'-^{32}P] pCp
3 ul clean oligonucleotide
2 ul 0.1mM ATP
4 ul 5X rx buffer*
10 ul H_2O for a 20ul reaction volume
1 ul RNA ligase (4ug/ul)[3]

Incubate for 90' at 30°, or 20hr at 4°.

Quench by adding 2.2ul 3M NaAcetate and 44.4ul EtOH, can also add 1 ug tRNA as carrier; precipitate (cold).

Re-precipitate with 250ul 0.3M NaAcetate, 750ul EtOH.

Wash pellet carefully with 95% cold EtOH; dry briefly under vacuum.

*RNA ligase 5X rx buffer:0.27M HEPES pH8.3, 0.1M $MgCl_2$, 0.024M DTT, 30% (v/v) deionized DMSO, 1% (w/v) BSA.

3' labelling with CTP and Terminal Deoxynucleotidyl Transferase (TdT)[4]:

cold $(pC_{ribo})_n$ tail:	hot $(pC_{ribo})_n$ tail:
10ul 500uM CTP	lyophilized [å^{32}P]-CTP
2ul clean oligonucleotide	2ul clean oligonucleotide
10ul 5X rx buffer*	10ul 5X rx buffer*

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[2] Krupp G. and H.J. Gross (1983) "Sequence analysis of in vitro ^{32}P-labelled RNA" in The Modified Nucleosides of Transfer RNA II, P.F. Agris (ed.) New York, pp11-58.

[3] BRL lot #41105

[4] a. *Methods in Enzymology* **65**:43-62, 508, 510,512, 523, 489. Especially see p46: To optimize tailing, use 10:1 rNTP:DNA ends, use ~.2 units TDT per pmole DNA ends.
CTPribo is the preferred nucleotide: generally 2 are incorporated, incorporation usually plateaus in 1-8 hrs. Assay by acid precipitable counts.

b. See also Amersham procedure I, with [alpha^{32}P]CTP.

H_2O for 50ul reaction volume	H_2O for 50ul reaction volume
3ul TdT	3ul TdT

Incubate overnight at 37°C.

Precipitate 2X, wash with 95% EtOH 1X, dry briefly under vacuum.

*TdT 5X rx buffer: 500mM potassium cacodylate, pH7.2, 10mM $CoCl_2$, and 1mM dithiothreitol.

After reaction, add 1ug tRNA carrier, ammonium acetate to .3M and 2vols EtOH, ppt; reppt w/ 250ul NaAc, 750ul EtOH; wash gently w/EtOH, cfg to settle pellet, remove super, dry.

Use [å-^{32}P]dideoxy NTP for internal labelling with no increase in charge at the terminus.

Cleavage of 3'-tail, 3'-p*$(rC)_n$, to single 3'-p*rCp* with pancreatic Ribonuclease A*:

Bring up precipitated 3'-tailed oligonucleotide in 400ul TES(10:1:50, pH7.2), add:

1ul panc. RNase A
10mg/ml stock, DNase free

Mix, incubate 30' at 37°.

Precipitate.

*Enzyme must be DNase free: boil for several minutes, slowly allow to cool to renature RNase. Stock conc = 10mg/ml. Use at 2.5ug/ml.

5' labelling with ATP and Polynucleotide Kinase[5]:

Denature oligonucleotide:

bring up dephosphorylated oligonucleotide in 40 ul H_2O
and 4 ul buffer A*

[5] Maniatis T., E.F. Fritsch, J. Sambrook (1982), in Molecular Cloning: a laboratory manual, Cold Spring Harbor, p.124.

Heat to 90°, chill quickly on ice. Add:

5 ul rx buffer B*
5 ul ATP; at least equimolar for maximum incorporation, either cold or [gamma-^{32}P] labelled.
20 units T4 Polynucleotide Kinase

Mix and incubate 30-45' at 37°.

Quench with 2 ul 0.5M EDTAS. Remove enzyme by phenol/chloroform extraction, with one back extraction; precipitation with 50 ul 7.5 M Ammonium Acetate, 300 ul EtOH, then wash with cold (-20°C) 80% EtOH. Purify by mini-chromatography on a NACS-prepac™.[6]

* Buffer A: 200 mM Tris-Cl (pH 9.5), 10 mM Spermidine, 1 mM EDTA.
Buffer B: 0.5 M Tris-Cl, 0.1 M $MgCl_2$, 50 mM dithiothreitol, 50% glycerol.

Dephosphorylation of DNA:

Protocol 1.[7]

dissolve DNA in min. vol.10mM Tris(8.0), and add:

5ul 10X CIP rx buffer*
H_2O to 48ul
0.01unit/pmole 5'-ends Calf Intestinal Phosphatase (CIP)

For protruding 5'-ends, incubate 30' at 37°; add 2nd aliquot enzyme and repeat incubation.

For blunt or recessed ends, incubate 15' at 37°, and15' at 56°; add 2nd aliquot CIP and repeat incubation.

To quench and purify: add 40ul H2O, 10ul 10X STE(100mM Tris.Cl (8.0), 1M NaCl, 10mM EDTA) and 5ul 10% SDS. Heat to 68°for 15'.

Phenol/chloroform extract 2X, chloroform extract 2X;

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[6] Bethesda Research Laboratories product, see instruction manual for further details. Alternatively, gel permeation chromatography can be used to remove small contaminating molecules, e.g. Maniatis T., et al, p.466.

[7] Ibid, pp.133-134.

Remove small molecular weight contaminants by Sephadex G-50 chromatography, then EtOH precipitation.

*10X CIP buf: 0.5 Tris.HCL(9.0), 10mM MgCl2, 1mM ZnCl2, 10mM spermidine.

Protocol 2.[8]

Bring up carrier tRNA (1 ug) and labelled oligonucleotide in 7ul water, add:
1ul 1M Tris-HCl pH8.0

Heat 2' at 100°C; chill on ice; spin down, add:
2 ul (10u/ml) CIP

Incubate1hr at 50°C. Spin down, add:
3ul nitrilotriacetic acid (NTA) pH7.2

Mix, incubate 20' at 50° to inactivate the phosphatase. Store if necessary at -20° or precipitate with cold NaAc/EtOH.

Protocol 3.[9]

Bring up clean DNA in 98ul water, add:
1ul Tris-HCl (8.0)

Heat to 65°, add:
3ul BAP (ammonium sulfate suspension)[10]

Incubate 45' at 65°

Stop reaction by phenol/chloroform extraction1X, back extract 1X with 5mM EDTA, precipitate.

Cleavage of 3' terminal ribose

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[8] Krupp G. and H.J. Gross (1983) "Sequence analysis of in vitro ^{32}P-labelled RNA" in The Modified Nucleosides of Transfer RNA II, P.F. Agris (ed.) New York, pp11-58.

[9] West, B., University of California, S.F., personal communication.

[10] see Maniatis T., E.F. Fritsch, J. Sambrook (1982), in Molecular Cloning: a laboratory manual, Cold Spring Harbor, p134 for preparation instructions if $(NH_4)_2SO_4$ is a problem, (e.g. it inhibits PNK).

Periodate oxidation:

Bring up precipitated oligonucleotide in 100ul NaAc(0.15M, pH adjusted to 5.3 with HOAc)[11] May also add 1ug tRNA as carrier. Add:
5ul 1mM (use approximately 100X excess);

Incubate on ice, 30', in the dark.

Quench and clean up by precipitation: add 5.6ul 3M NaAc, 2 vols EtOH; second precipitation; wash 1X w/ 80% EtOH. Dry briefly under vacuum.

Alkali cleavage of dialdehyde:

Dissolve oxidixed oligonucleotides from above with 100ul 1M piperidine, seal tubes and incubate at 95°, 30'. Lyophilize 3X to remove piperidine. Test results by analytical PAGE.

Analytical PAGE of oligonucleotides

Modifications to standard methods have been made for self complimentary (hairpin) oligonucleotides. These retain secondary structures, under normal sequencing gel conditions, that impair reading the sequence.

Formamide sequencing gels:[12]

To 25.5ml 25% stock PA/Urea, add 25%(8.5ml deionized) formamide, to give 20% final gel concentration.

Pre-warm 1X TBE and polymerized gel (>2 hours). Load samples normally.

Fix gel: remove one plate, soak gel in 10% MeOH, 10% Acetic Acid for 10 minutes only (gel swells), aspirate off solution., adsorb gel to 3MM paper, blot this if necessary, must dry in gel dryer unless its so hot that only a brief

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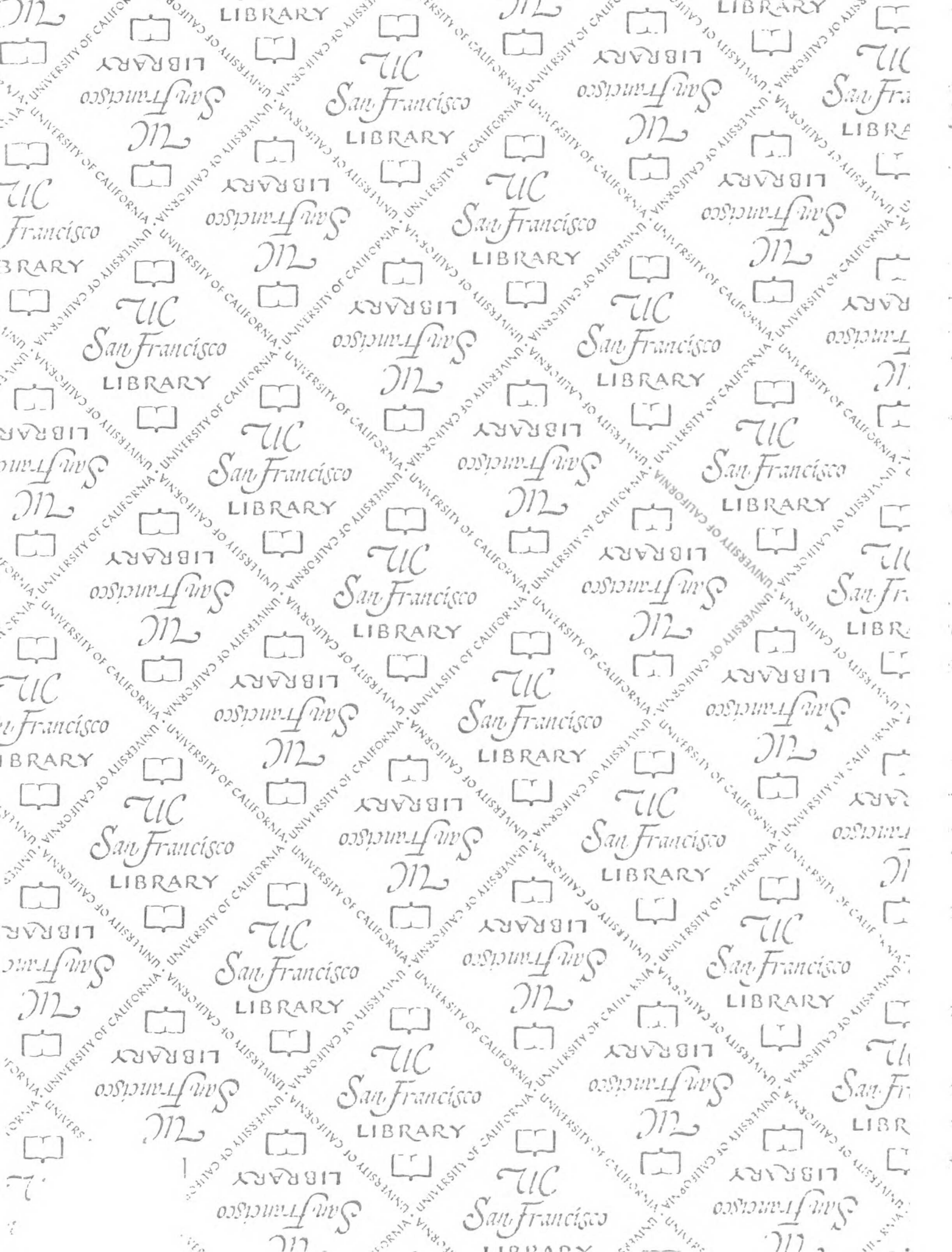
[11] Slightly acidic conditions are used to limit dehydration of the dialdehydes to a cyclized species; N.J. Oppenheimer, personal communication.

[12] Mary Betlach, U.C.S.F. (H. Boyer's lab) personal communication, see also Bio-Rad Bulletin.

exposure is necessary, for ^{35}S autoradiography may be done without saran wrap.

Preparative PAGE:

Gel is 20% polyacrylamide, 7.5 M urea. Samples are brought up in water, add 1/5 total vol. 70% glycerol. Electrophorese a 2mm thick gel 12 hours at 500-700V. Mark band using UV light/fluoresc. TLC plate; cut out; soak O/N in H_2O; filter: dispo. 0.45u (Gelman Acrodisc), collect in SW41 pollyallomer tube (11mls vol); add NH_4Ac to 2M; add 2 vols EtOH; ppt -20 (may add1ug cold tRNA carrier to labelled samples). Centrifuge 30K, 30'; discard super; dry; bring up in H_2O; transfer to eppendorf tube.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