# Isotope Effects on the Cleavage of DNA by Bleomycin: Mechanism and Modulation†

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ABSTRACT: Sequence-specific isotope effects on the cleavage of DNA at thymidine residues by activated iron·bleomycin (Fe·BLM) have been observed upon incorporation of [4′-$^2$H]thymidine into the DNA. The effects may be quantitated by end-labeling the DNA with phosphorus-32, generating a sequence ladder by gel electrophoresis, and measuring the relative damage at cleavage sites by autoradiography or phosphorimager technology. Results with DNA deuteriated at other positions of the deoxyribose ring, such as, the 5′- or 2′-carbon, afford no isotope effect suggesting high regiospecificity for the BLM-mediated carbon–hydrogen bond cleavage. The magnitude of the 4′-isotope effects for Fe·BLM ranges from 2 to 7 (±0.3) and is not sensitive to changes in the partition ratio of the two proposed cleavage pathways. However, the isotope effect is sensitive to structural changes in BLM, such as with tallysomycin, and to changes in metal and cleavage mechanism, such as with Co·BLM. The variability of the effects are discussed in light of the factors responsible for the observation of $V/K$ isotope effects. The findings suggest that the kinetics, thermodynamics, and geometry of carbon–hydrogen bond cleavage by Fe·BLM permit the observation of large isotope effects on this chemical step. Fe·BLM may be viewed, therefore, as a special case.

The elucidation of the mechanism of action of drugs that cleave DNA via the homolysis of carbon–hydrogen bonds in the deoxyribose moiety has been a long term goal of our laboratories. Bleomycin (BLM;[1] Figure 1) has served as the prototypic DNA cleaver (Stubbe & Kozarich, 1987). Our initial investigations focused on the mechanism of cleavage of a DNA model, poly(dA·dU), by Fe·BLM. The copolymer was tritiated at specific positions of the deoxyribose of the deoxyuridine residues in order to determine which carbon–hydrogen bonds were labilized during the cleavage process (Wu et al., 1983, 1985a,b). The results led to the hypothesis that DNA damage by activated Fe·BLM was initiated by a homolytic cleavage of the 4′-carbon–hydrogen bond and that this homolysis was subject to a large tritium selection effect ($k_H/k_T$ = 7–11). In addition, our findings were consistent with the proposal that both damage pathways (A and B; Scheme I) originated from this highly specific, drug-mediated event. Pathway A leads to DNA strand scission under neutral conditions with the formation of a 3′-phosphoglycolate terminus and the nucleic acid base propenal. Pathway B affords an alkali-labile lesion via a $\beta$-elimination that ultimately produces a 3′-phosphate terminus by some complex chemistry that remains to be elucidated. The partitioning between these two pathways as expected could be modulated by the availability of $O_2$. The similarity of tritium selection effects under several aerobic and anaerobic conditions supported the hypothesis that both pathways are initiated by 4′-carbon–hydrogen bond cleavage and, thus, the products are derived from a common intermediate.

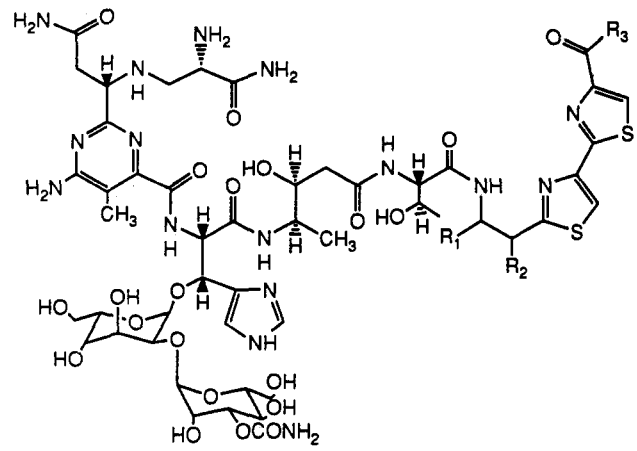


FIGURE 1: Structure of bleomycin (BLM) and tallysomycin (TLM).

The observation of large tritium selection effects suggested that the incorporation of specifically 4′-deuteriated deoxynucleotides of high isotopic content into DNA of defined sequence could permit the direct measurement of isotope effects on Fe·BLM-induced cleavage at sequence sites bearing deuterium using DNA sequencing technology (Kozarich et al., 1989). A $^{32}$P end label is used as a remote label to quantitate the effect. We have initially evaluated this method with Fe·BLM (Kozarich et al., 1989) and with the neocarzi-

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[1] Abbreviations: ALM, alkaline labile moiety; BLM, bleomycin; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; SDS, sodium docecyl sulfate; TLM, tallysomycin.

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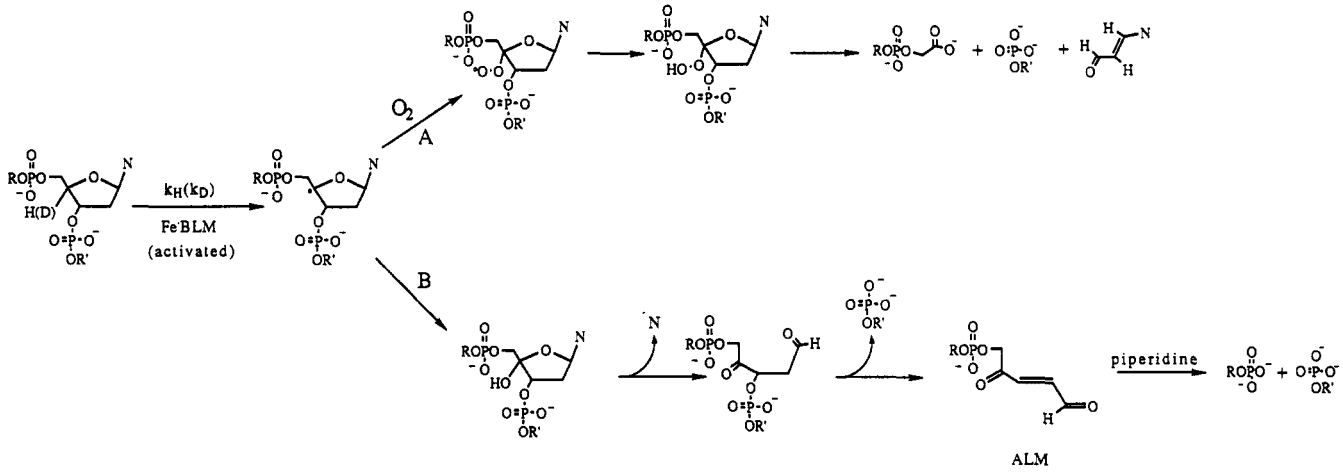
Scheme I: Mechanism of DNA Cleavage by Fe·BLM[a]




[a] Pathways A and B partition from a common intermediate which is generated by 4′-carbon hydrogen bond cleavage. Pathway A is oxygen-dependent and leads to the formation of base propenals and 3′-phosphoglycolate termini. Pathway B is oxygen-independent and leads to the initial formation of a 4′-hydroxyl species and nucleic acid base. A β-elimination from this species yields an ALM fragment which is further decomposed upon more vigorous alkaline conditions to afford 3′-phosphate termini.

*nostatin* chromophore (Kappen et al., 1990, 1991; Frank et al., 1991).

In this paper we define the basis of this isotope effect methodology and its inherent scope and limitations. Using as an example the DNA cleavage by Fe·BLM with its specificity for 4′-carbon–hydrogen bond cleavage, we provide an initial assessment of the modulation of the isotope effect by drug structure and by local sequence environment.

## MATERIALS AND METHODS

Blenoxane (bleomycin; BLM) and tallysomycin (TLM) were gifts of Bristol-Meyers Squibb. Blenoxane is a mixture of $A_2$ and $B_2$ BLM and was used without purification. Deuteriated $NaBH_4$ (99% atom D) and deuteriated diborane ($BD_3$·THF, ~1 M) were purchased from Norell, Inc. and Alfa, respectively. [4′-$^2$H]Thymidine 5′-triphosphate was prepared as previously described (Ajmera et al., 1986a). [5′,5′-$^2H_2$]Thymidine 5′-triphosphate was prepared as previously described (Frank et al., 1991). [2′-(*R*)-$^2$H]dCTP was prepared from CTP by enzymatic reduction in $D_2O$ using ribonucleotide reductase from *Lactobacillus leichmanii* (Wu et al., 1985a). [γ-$^{32}$P]Adenosine 5′-triphosphate (>5,000 Ci/mmol) was purchased from Amersham. Deoxynucleoside 5′-triphosphates and Sephadex G-50 were obtained from Pharmacia P-L Biochemicals. All other chemicals were of reagent grade and came from Aldrich.

T4 Polynucleotide kinase (10 units/μL) and the M13 –40 sequencing primer (17-mer) were purchased from New England Biolabs. T4 DNA ligase (2 units/μL), restriction endonucleases (*Eco*RI, *Bam*HI, and *Hin*dIII; 10 unit/μL), and NACS-52 Prepac columns were obtained from Bethesda Research Laboratories. Calf intestinal phosphatase (CIP; 1 unit/μL) was purchased from Boehringer-Mannheim. Sequenase (12.5 units/μL) was obtained from United States Biochemical Corp. *Escherichia coli* JM101, pBR322, and M13mp19 were gifts of Professor John Gerlt, University of Maryland. Electrophoresis reagents were from Research Organics and silica gel GF TLC preparative plates (1000 μm) were from Alltech. Centricon (C-10) microseparation filters were purchased from Amicon. Anaerobic reactions were performed in screw cap septum vials (1.5 mL) obtained from Pierce.

*Preparation of Single-Stranded DNA Template.* The 375 bp *Eco*RI/*Bam*HI fragment from pBR322 was ligated into M13mp19, and the phage was harvested from cultures of JM101 by established procedures (Maniatis et al., 1982). Isolation and purification of the ssDNA was accomplished by the methodology of Sayers and Eckstein (1987). This procedure usually produced ~1 mg of ssDNA from a 300-mL growth.

*Preparation of (+) Strand of M13 Containing [4′-$^2$H]-Thymidine.* The –40 primer of the (+) strand (150 pmol) in 150 mM Tris-HCl (35 μL, pH 8.0), containing 10 mM $MgCl_2$, 5 mM DTT, 1 mM ATP, and T4 kinase (10 units) was incubated at 37 °C for 30 min. The single-stranded M13 template with the pBR322 insert (30 pmol) in 140 mM Tris-HCl (225 μL, pH 8.0) containing 140 mM NaCl was incubated at 70 °C for 5 min and at 37 °C for 20 min and then placed on ice prior to double-strand polymerization.

A typical polymerization reaction (250 μL) in 190 mM Tris-HCl (pH 8.0) contained 80 nM template–primer, 0.3 mM each of dGTP, dATP, dCTP, and [4′-$^2$H]TTP (or bis-[5′,5′-$^2$H]TTP), 1.2 mM ATP, 12 mM $MgCl_2$, and both Sequenase (50 units) and T4 ligase (37 units). Incubation was carried out at 37 °C for 1 h. A second equivalent of T4 ligase (37 units) was then added and the reaction incubated at 16 °C for 16 h. The sample was quenched by the addition of 0.5 M EDTA (16 μL) and heated to 70 °C for 10 min. The solution was extracted twice with an equal volume of phenol/$CHCl_3$/isoamyl alcohol (24:24:1), washed with ether (3 × 300 μL), and precipitated by established procedures (Maniatis et al., 1982). Purification was accomplished by centrifugation using Centricon (C-10, 10 000 MW cutoff) desalting cellulose filters (4 × 1-mL $H_2O$ washes). Approximate concentration of double-stranded DNA obtained by this method ranged from 1.5 to 2 μg/μL (~80–90% yield). All samples were analyzed on an 0.8% agarose gel. No single-stranded DNA was observed, and >90% of the material exhibited a mobility consistent with closed circular DNA. The T4 ligation step was included in order to minimize extraneous ends during subsequent phosphatase and kinase treatment.

The dsDNA was digested with *Eco*RI to expose the pBR322 5′-overhang end of the (+) strand, and the linearized material was dephosphorylated at the 5′-ends by the action of CIP. The sample was extracted and precipitated as described above. The 5′-$^{32}$P-end-labeling of the dephosphorylated DNA was accomplished with T4 polynucleotide DNA kinase (New England Biolabs, BRL). A typical reaction (25 μL) in 50

mM Tris-HCl (pH 7.5) contained 10 mM $MgCl_2$, 5 mM DTT, the dephosphorylated DNA fragment (~10 μg; 2 pmol), BSA (10 μg/μL), [γ-$^{32}$P]ATP (250 μCi, ~80 pmol), and T4 kinase (25 units). Incubations were carried out at 37 °C for 45 min. The kinase reactions were then terminated and precipitated with 7.5 M ammonium acetate ($NH_4OAc$; 30 μL) and cold (–20 °C) ethanol (300 μL). The samples were placed in a dry ice/acetone bath for 1 h and sedimented by centrifugation for 10 min. This centrifugation step was repeated with 80% cold ethanol (250 μL). Pellets were carefully dried and resuspended in $H_2O$ (25 μL).

The 5′-$^{32}$P-end-labeled DNA was digested with *Bam*HI to release the 375 bp pBR322 fragment. The restriction digest in a total volume of 30 μL in 50 mM Tris-HCl (pH 8.0) contained 10 mM $MgCl_2$, 100 mM NaCl, $^{32}$P-labeled DNA (~5–10 μg), and *Bam*HI (10 units). Reactions were incubated at 37 °C for 2 h and terminated by addition of 0.5 M EDTA (5 μL; pH 8.0). Samples were lyophilized to dryness and resuspended in nondenaturing buffer (15 μL; 50% glycerol, 0.1% w/v xylene cyanol, 0.1% w/v bromophenol blue, 1 mM EDTA). The 375 bp fragment was purified by electrophoresis on a 5% disulfide-cross-linked, polyacrylamide gel according to the procedure of Hansen (1976) followed by chromatography on a NACS column.

*Symmetric Polymerase Chain Reaction (PCR).* A PCR method of preparing deuteriated DNA fragments has also been utilized. A typical reaction (100 μL) in 10 mM Tris-HCl (pH 8.3) contained 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, 200 μM of each deoxynucleoside 5′-triphosphate (including the specifically deuteriated nucleotide), 1 μM each of the two primers directed to the fragment, 2.5 units *Taq* polymerase, and 1 ng of target DNA in 100 μL. An Eppendorf Microcycler was programmed as follows for amplification: the DNA was denatured by heating to 94 °C for 1.5 min and then cooled to 37 °C for 2 min to anneal the primers to the template. A three-step cycle was repeated 30 times, which consisted of 5 min at 74 °C, 1 min at 94 °C, and 2 min at 37 °C. After the last cycle, the sample was held at 74 °C for 30 min. This method results in the incorporation of deuteriated nucleotide into both stands with the exception of the primer regions. Dilution of deuterium content in the amplified fragment (yield ~10 μg) by original unlabeled template (1 ng) is not significant.

*Asymmetric PCR.* The (–) strand of a 77 bp fragment of the *nrd*A gene contained in M13mp18 was perferentially amplified over the (+) strand by asymmetric PCR and purified from unincorporated dNTPs using conventional protocols (Gyllensten & Erlich, 1988). In a separate reaction vessel, 1 pmol of 5′-$^{32}$P-labeled (+) primer was annealed to ~0.5 pmol of the asymmetric PCR product and the (+) strand filled in using the Klenow fragment of DNA polymerase in the presence of 200 μM each of [2′-(*R*)-$^2$H]dCTP, dATP, dGTP, and TTP. Control DNA was prepared using unlabeled dCTP in place of the deuteriated dCTP. The $^{32}$P-labeled double-stranded 77 bp fragment was purified using nondenaturing polyacrylamide gel electrophoresis and isolated from the gels using standard procedures.

*Fe·Bleomycin Reactions.* Three different reaction conditions were employed:

(*1*) *Normal Atmosphere.* A typical reaction (200 μL) contained the labeled fragment (~300 000 cpm), 50 mM Tris-HCL (or 10 mM sodium phosphate, pH 7.5), 3.5 μM bleomycin or tallysomycin, salmon sperm DNA (~200 μg/mL), and 9.0 μM ferrous sulfate. The reactions were initiated by the addition of ferrous sulfate (freshly prepared). The reaction was permitted to proceed at room temperature for 10 min. An excess of salmon sperm DNA (400 μg/mL) and 10 mM EDTA was subsequently added to the reaction followed by 2.5 M NaOAc (0.25 M final). The solution was immediately precipitated with two volumes of cold ethanol (–20 °C).

(*2*) *Anaerobic.* Anaerobic reactions conditions followed the general procedures of Burger et al. (1981) and of Wu et al. (1983, 1985a). A typical reaction (200 μL) contained the labeled fragment (~300 000 cpm), 10 mM sodium phosphate (pH 7.5), 6 μM bleomycin or tallysomycin, and 5 μM ferric ammonium sulfate. The reaction was sealed in a screw cap septum vial (1.5 mL) at room temperature and constantly purged with $N_2$ bubbled through Fieser's solution (Fieser & Fieser, 1967) for 30 min. The reaction was initiated with 400 μM $H_2O_2$ ($N_2$-purged) to give a final concentration of 88 μM and allowed to proceed at room temperature for 15 min. Workup of the reaction was performed anaerobically using the same methodology as that used for the normal atmosphere reaction (vide supra). The sample was divided into two Eppendorf tubes and precipitated.

(*3*) *Elevated $O_2$ Pressure.* The protocol was essentially identical to that described for normal atmospheric conditions except that the reaction was performed in a 1.5-mL Eppendorf tube placed in a modified 10-mL Amicon Model 12 filtration chamber (Wu et al., 1985a). The chamber was fitted with a Hamilton syringe for the addition of the ferrous sulfate solution and with a 26S gauge needle (0.114-mm i.d.) that permitted constant stream of $O_2$ to pass through the reaction solution at elevated (>3 atm). The reaction (200 μL) was initiated by the addition of ferrous sulfate and maintained at 4 °C for 10 min. Termination and precipitation of the reaction were performed as described above.

Samples (200 μL) from all sets of reaction conditions were usually divided in half prior to precipitation, and one portion was subjected to piperidine/heat treatment. The precipitated DNA was treated with 1 M piperidine (100 μL) at 90 °C for 30 min, quenched with a solution of 1 M acetic acid and 0.5 M NaOAc (200 μL), and precipitated with cold ethanol (2 vol). Upon being washed with cold ethanol and drying, the pellet was resuspended in denaturing loading buffer (6 μL; 95% formamide, 10 mM EDTA, 0.1% w/v xylene cyanol FF, 0.1% w/v bromophenol blue).

*Co·BLM.* A typical reaction (40 μL) in 50 mM Tris-HCl (pH 7.5) contained the labeled fragment (180 000 cpm), 9.5 μM bleomycin, 26 μM $CoCl_2$, and salmon sperm DNA (10 μg) in a 1.5-mL Eppendorf tube. Reactions were initiated by irradiation with a germicidal lamp for 10 min at 0 °C. Samples were precipitated by adding 0.3 M sodium acetate (200 μL), sonicated salmon sperm DNA (40 μg), and cold 100% ethanol (750 μL). The DNA was sedimented by centrifugation, resuspended in cold 70% aqueous ethanol (1 mL), sedimented by centrifugation, and dried in vacuo. For alkali-treated samples, the dried pellet was treated as described for Fe·BLM reactions.

*Analysis.* Reactions samples were analyzed on a 20% denaturing polyacrylamide gel that was pre-electrophoresed in TBE buffer (100 mM Tris, 100 mM boric acid, and 2 mM sodium EDTA, pH 8.3) at 2700 V (~45 °C) for 1 h. All samples were heat denatured in loading buffer at 90 °C for 3 min and then chilled on ice for 2 min. Equal amounts of radioactivity for each reaction sample were loaded and subjected to electrophoresis at 2700 V for 3 h. Maxam/Gilbert sequencing reactions were run as standards (Maxam & Gilbert, 1980). The gel was removed and dried on Whatman

filter paper. Peak volume quantitation was performed on a Molecular Dynamics 400A PhosphorImager using the ImageQuant program. The procedure for the quantitation and normalization of peak areas has been previously described (Frank et al., 1991). The gel was also subjected to autoradiography at room temperature with Kodak X-OMAT AR double-sided film (~30 h).

## RESULTS AND DISCUSSION

*Analysis of DNA Cleavage Using Tritium.* Our work on BLM-mediated cleavage of DNA models, such as poly(dA·[$^3$H]dU) tritiated at specific deoxyribose positions, required the careful quantitation of tritiated products (Wu et al., 1983, 1985a,b). For instance, the amount of tritiated water produced was used as an indicator for carbon–hydrogen bound cleavage (homolytic or heterolytic) at a particular carbon. Tritium was also quantitated in reisolated starting material (Wu et al., 1985a) and in products derived from the cleavage process (Ajmera et al., 1986b). Using a variety of conditions, we were able to establish which hydrogens appeared to be labilized by heterolytic, chemical processes that occurred after the BLM-mediated event. The large selection effect measured in the case of [4′-$^3$H]dU with activated Fe·BLM and the absence of significant chemical exchange from this position in the damaged DNA strongly suggested that cleavage of the 4′-carbon–hydrogen bond is the unique, BLM-mediated event that leads to the products formed in both pathways (Scheme I, pathways A and B). A number of studies using defined oligomers and model compounds have been performed to elucidate the structure of the alkali-labile product and the source of oxygen incorporation into products (Rabow et al., 1986, 1990a,b; McGall et al., 1987, 1991, 1992). On the basis of precedent with other systems involving iron–oxygen chemistry, such as cytochrome $P_{450}$, the 4′-carbon–hydrogen bond cleavage is most reasonably homolytic although this remains to be rigorously established.

There are a number of disadvantages to the use of tritium in these experiments. First, the difficulty encountered in the multiple-step syntheses required for the preparation of labeled nucleotides limits the specific activity that can be attained on a practical laboratory scale. Second, relatively small errors in the determination of the extent of DNA damage by product quantitation can lead to large errors in the quantitation of a large selection effect (Wu et al., 1985a). Third, the tritium selection effects do not provide information on sequence dependence in a large heterogeneous DNA since only global product formation is measured.

*Analysis of DNA Cleavage with Deuterium.* The Swain–Schaad equation predicts that a tritium selection effect of 7–11 will result in a corresponding deuterium effect of ~4–5.5 (Swain et al., 1958). This analysis suggested that a deuterium isotope effect could be detected at individual sequence sites in heterogeneous DNA under single hit conditions (Kozarich et al., 1989). The deuteriated nucleotide (such as [4′-$^2$H]thymidine triphosphate) of high isotopic content may be incorporated into a defined double-stranded DNA fragment using a number of procedures. The deuteriated strand to be analyzed is 5′-end-labeled with $^{32}$P to serve as the remote label for quantitation of damage with high sensitivity.

In a typical reaction, the doubly labeled DNA is treated with a limiting amount of Fe·BLM in order to generate a set of damage fragments that can be identified by polyacrylamide gel electrophoresis and quantitated by autoradiography and phosphorimager technology. The relative distribution of fragments on the gel is a reflection of the sequence specificity of the Fe·BLM and, therefore, of the relative rates of carbon–hydrogen (deuterium) bond cleavage. The amount of damage at deuteriated residues will be suppressed relative to an unlabeled DNA control if there is an isotope effect on the cleavage. Thus, the effects at discreet sequence sites can be directly visualized and quantitated.

The observation of the isotope effect is an informative and fortunate occurrence since the basis for its observation is complex and many factors can attenuate its manifestation. If, for example, damage at a given site is the result of a specific carbon–hydrogen bond cleavage (e.g., the 4′-hydrogen), then a unique isotope effect *may* be expressed on the fragment(s) generated by cleavage at that site. If, however, a number of hydrogens bound to a deoxyribose are susceptible to homolysis, the isotope effect may be measured *only* if that specific carbon–hydrogen(deuterium) bond cleavage leads to the production of a uniquely observed fragment. For example, hydrogen abstraction at virtually every deoxyribose position ultimately can lead to the formation, by different mechanisms, of fragments terminating in 3′-phosphate. Therefore, while deuteriation at one position may suppress the formation of 3′-phosphate fragments (equivalent to the Maxam/Gilbert fragments), the internal kinetic partitioning of activated bleomycin to another susceptible hydrogen will also eventually generate the same fragment, thereby attenuating the observed suppression. However, 4′-hydrogen abstraction has been shown in a number of systems to *uniquely* afford fragments with 3′-phosphoglycolate termini (Scheme I, pathway A) as well as 3′-phosphate termini (Scheme I, pathway B). In the case of bleomycin, this is based on extensive studies with model oligomers that included isolation, characterization, and quantitation of products (vide supra). High-resolution gel electrophoresis (20% cross-link) can easily separate 3′-phosphate and 3′-phosphoglycolate termini for small fragments (<30 bp) derived at the same sequence site. Thus, quantitation of the suppression of 3′-phosphoglycolate fragments can be used to determine isotope effects at the 4′-position even if other positions are susceptible to cleavage chemistry.

Assuming a complete positional specificity for hydrogen abstraction, the magnitude of the observed isotope effect will be affected by a number of factors. One of these is the relative rate of partitioning of the activated BLM·DNA complex between hydrogen (deuterium) abstraction ($k_H$ or $k_D$) and nonproductive processes ($k_X$). These nonproductive events can include disassociation of activated BLM from the DNA ($k_{dissoc}$), sliding of the activated BLM in the minor groove away from the cleavage site ($k_{slide}$), and self-destruction of activated BLM while bound to DNA ($k_{inact}$). The sum of these processes ($k_X = k_{dissoc} + k_{slide} + k_{inact} + ...k_n$) constitutes the pathways available for isotopic discrimination at a given sequence site. Thus, the deuterium isotope effects determined by this methodology are selection effects [$^D(V/K)$]: a limited concentration of BLM must discriminate among an excess of cleavage sites, some of which are deuteriated. The simplest kinetic model, then, predicts that the selection effect be described by

$$^D(V/K) = \frac{k_H}{k_D}\frac{(k_D + k_X)}{(k_H + k_X)} \quad (1)$$

where $k_X$ is the total rate constant for nonproductive processes of the activated BLM·DNA complex at the nucleotide site in question and $k_H$ and $k_D$ are the rate constants for carbon–hydrogen and carbon–deuterium bond cleavage at that site, respectively. Clearly, the rates of BLM activation and drug binding to DNA are not factors, and it is reasonable to assume

that $k_X$ is not affected by deuteriation at the DNA residue. A large observed isotope effect ($k_H/k_D$) is possible, then, only when $k_X \gg k_{H(D)}$.

In the parlance of the enzymologist, this type of isotopic discrimination requires a low commmitment to catalysis (abstraction) and will be observable only if the isotopically sensitive step occurs before, or is, the first irreversible step in the reaction. This can be illustrated by considering the two extreme cases. In the case of high commitment to cleavage ($k_X \ll k_{H(D)}$), the binding of activated BLM to DNA may be considered irreversible and isotope transparent. Thus, discriminatory processes ($k_X$) are slower than abstraction ($k_{H(D)}$) and there is no observable isotope effect. Conversely, for low commitment to cleavage ($k_X \gg k_{H(D)}$), an isotope effect can be observed since discriminatory processes are kinetically significant. It is of utmost significance that $k_H$ and $k_D$ are *not* required to be rate-determining for the overall reaction; that is, the isotope effect need not be manifested on $V_{max}$ where the rate of binding is suppressed by saturating DNA with BLM. Under $V_{max}$ conditions, it is conceivable that a later step, such as peroxysugar fragmentation in phosphoglycolate production (Scheme I, pathway A), is rate-determining. Low commitment to cleavage is, therefore, the most important determinant in the expression of the intrinsic isotope effect ($k_H/k_D$).

However, the intrinsic magnitude of $k_H/k_D$ itself may be attenuated by other factors. Most important among these are the extents of nonlinearity and of asymmetry in the transition state of the abstraction step (Westheimer, 1961; More O'Ferrall & Kouba, 1967; More O'Ferrall, 1970). Maximal isotope effects are observed for linear, symmetric transition states. The structure of activated BLM, the local DNA conformation (e.g., sugar pucker), and the overall structure of the resulting activated BLM·DNA complex will be factors in the trajectories of the susceptible carbon–hydrogen bond and the putative Fe–O bond in activated BLM. The symmetry of the transition state will be affected by differences in the reactivity of activated BLM and the resultant nucleotide radical. A large disparity in the reactivity of these species leads to asymmetry in the transition state of hydrogen (deuterium) transfer and a corresponding attenuation of the intrinsic isotope effect.

In summary, a maximal isotope effect on DNA cleavage will be observed if the hydrogen abstraction is a regiospecific, low commitment process that proceeds via an in-line, symmetric transition state. Deviations from these parameters will lead to smaller or no observed isotope effects and constitute the bases for modulation of the isotope effect at individual sequence sites.

*Fe·BLM Reactions.* In light of the above discussion, we now consider the analysis of the cleavage of DNA containing [4′-$^2$H]- or [5′,5′-$^2H_2$]thymidine by Fe·BLM (Figure 2). The autoradiogram of the reaction under normal atmospheric $O_2$ confirms the presence of a substantial suppression in the amount of *all* DNA fragments associated with cleavage at thymidine upon 4′-deuteriation. No significant effect is seen upon 5′-deuteriation (Table I). The error in the measurement of the isotope effects is generally ±0.3. Thus, for large effects the error is quite low (±10%). Under neutral conditions (Figure 2, lanes 1–3), 3′-phosphoglycolate-terminating fragments are observed as well as a slower moving fragment bearing an alkaline labile moiety (ALM). Upon treatment with piperidine, the ALM fragments are converted to the faster-moving fragments terminating in 3′-phosphate (Maxam/Gilbert fragments; lanes 4–6) resulting in the formation of

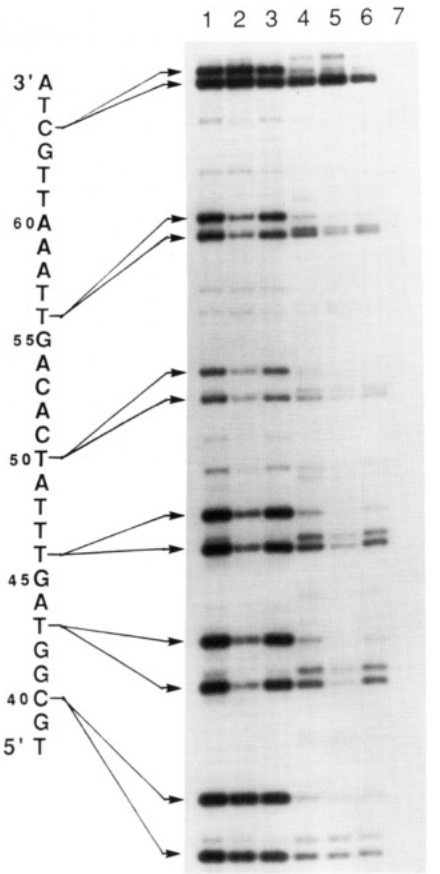



FIGURE 2: Isotope effects on the cleavage of DNA containing [4′-$^2$H]- or [5′,5′-$^2H_2$]thymidine residues by activated Fe·BLM under normal atmosphere. The 5′-$^{32}$P-end-labeled *Hin*dIII/*Bam*HI fragment of pBR322 containing thymidine (lanes 1 and 4), [4′-$^2$H]-thymidine (lanes 2 and 5), or [5′,5′-$^2H_2$]thymidine (lanes 3 and 6) was cleaved by Fe(II) and BLM as described under Materials and Methods, separated by electrophoresis on a 20% denaturing polyacrylamide gel, and subjected to autoradiography. Samples were analyzed prior to (lanes 1–3) and after (lanes 4–6) piperidine treatment. Doublets observed before piperidine treatment (lanes 1–3) are due to 3′-phosphoglycolate termini (faster moving band) and to 3′-termini containing an alkaline labile moiety (ALM; see Scheme I). Upon piperidine treatment (lanes 4–6) the ALM termini are converted to 3′-phosphate termini. Suppression of cleavage due to $V/K$ isotope effects is clearly seen in DNA containing [4′-$^2$H]-thymidine (lanes 2 and 5). Control DNA is shown in lane 7. Isotope effects were quantitated using a phosphorimager (see Table I).

close doublets at each cleavage site that is indicative of a nearly equal distribution of 3′-phosphate and 3′-phosphoglycolate fragments. Within experimental error, the 4′-isotope effects are identical for each of the three observed termini associated with cleavage at a specific thymidine residue. Some isotope effects appear to be close to the theoretical maximum (e.g., $k_H/k_D \sim 6$–7 for $T_{48}$; Table I).

The largest variability in the isotope effects is observed for the ALM-terminating fragments in the cleavage of DNA by Fe·BLM and by the neocarzinostatin chromophore (Frank et al., 1991). These fragments have not been generally observed in the BLM reaction since their formation and decomposition are sensitive to pH. Figure 2 shows that the ALM fragments are formed by 4′-hydrogen abstraction and are precursors to the 3′-phosphate fragments. Thus, they are most reasonably formed by β-elimination of the 3′-phosphate to afford an unsaturated ketoaldehyde (ALM, Scheme I). We have tentatively assigned the geometry of the ALM as trans on the basis of recent stereochemical studies of the alkaline-induced β-elimination at aldehydic abasic sites in DNA (Mazumder et al., 1991). The variability in the isotope effect for this

Table I: Isotope Effects on 4′- and 5′-Hydrogen Abstraction from Thymidine by Activated Fe·BLM on the *Hin*dIII/*Bam*HI Restriction Fragment of pBR322[a]

| residue[b] | terminus[c] | isotope effect[d] 4′ | 5′ |
|---|---|---|---|
| C-40 | P | 0.9–1.2 | 1.0–1.2 |
| | ALM | 0.7–1.0 | 0.8–1.3 |
| | PG | 1.0–1.4 | 0.9–1.3 |
| T-43 | P | 3.7–4.2 | 0.8–1.2 |
| | ALM | 3.2–4.1 | 0.7–1.2 |
| | PG | 4.0–4.6 | 1.0–1.3 |
| T-46 | P | 4.2–4.7 | 0.7–1.2 |
| | ALM | 3.6–4.4 | 0.9–1.4 |
| | PG | 3.9–4.2 | 1.1–1.2 |
| T-48 | P | 5.8–6.3 | 0.8–1.3 |
| | ALM | 5.5–5.9 | 0.7–1.0 |
| | PG | 6.4–7.2 | 1.0–1.2 |
| T-50 | P | 3.6–3.9 | 0.9–1.3 |
| | ALM | 3.3–4.0 | 1.0–1.2 |
| | PG | 3.3–3.7 | 1.1–1.4 |
| C-53 | P | 0.7–1.0 | 0.9–1.1 |
| | ALM | 0.9–1.4 | 0.7–1.1 |
| | PG | 0.8–1.2 | 1.0–1.1 |
| T-56 | P | 3.1–3.8 | 1.0–1.4 |
| | ALM | 3.4 | |
| | PG | 3.5–4.0 | 0.8–1.3 |
| C-65 | P | 0.8–1.1 | 1.1–1.3 |
| | PG | 0.8–1.0 | 0.9–1.3 |

[a] Reactions were carried out under normal atmospheric conditions. [b] Residues of the (+) strand of pBR322 are numbered from the *Eco*R1 site. [c] Fragments terminating in 3′-phosphate (P) and 3′-phosphoglycolate (PG) were quantitated separately as described under Materials and Methods. [d] Isotope effects are listed as the ranges of three independent reactions.

fragment appears to be due to variability in quantitatively generating this fragment from an alkaline-labile lesion (Scheme I, pathway B) by the chemical $\beta$-elimination of the 3′-phosphate. Thus, inefficient formation of the ALM fragment will effect the quantitation of this fragment and, therefore, the magnitude of the apparent effect. Since the formation of 3′-phosphate termini can be achieved quantitatively from either the alkaline-labile lesion or the subsequent ALM fragment, much less variability is observed.

The regiospecificity of the initial carbon–hydrogen bond cleavage has also been investigated using [2′-(*R*)-$^2$H]dC incorporated into the *E. coli nrd*A gene. The 2′-(*R*)-proton is known to be stereospecifically labilized during the formation of trans-base propenal and the 3′-phosphoglycolate terminus (Wu et al., 1985b). In addition, the formation of 3′-phosphate termini also results in labilization of this proton. Since deprotonation of the 2′-(*R*)-position occurs after 4′-hydrogen abstraction by activated BLM (the first irreversible step), the constraints of the observed $V/K$ isotope effect dictate that no 2′-(*R*)-isotope effect will be observed. The results indicated in Table II support this conclusion. The observations with 2′- and 5′-deuteriated dNTPs and our prevuous work with 1′-labeled dNTPs (Wu et al., 1983; Absalon et al., 1992) are consistent with high regiospecificity for 4′-hydrogen abstraction by BLM and with the mechanistic and kinetic implications of a $V/K$ effect.

Under conditions of high $O_2$ concentration (3 atm), a clear preference for the formation of fragments terminating in 3′-phosphoglycolate fragments is observed consistent with previous observations (Wu et al., 1985a; Figure 3A). Alternatively, anaerobic activation of Fe(III)·BLM with $H_2O_2$ affords, as expected, a shift toward the production of 3′-phosphate fragments after piperidine treatment (Figure 3B). The 4′-isotope effects are clearly observed for both sets of conditions and are essentially identical for a given thymidine residue. These data show that varying the availability of $O_2$ can change the ratio of 3′-phosphate and 3′-phosphoglycolate terminating fragments but does not change the magnitude of the observed isotope effect at a given residue. A common 4′-radical intermediate leading to both cleavage products is thus inferred. In addition, the equivalence of isotope effects demonstrate that activated Fe·BLM generated anaerobically with Fe(III) and $H_2O_2$ is chemically and mechanistically equivalent to activated Fe·BLM generated aerobically with Fe(II) and $O_2$. Previous studies have shown the spectroscopic equivalence of both activation procedures (Burger et al., 1981, 1983).

Table II: Isotope Effects on 2′-(*R*)-Hydrogen Abstraction from Cytidine by Activated Fe·BLM on the PCR Fragment of the *E. coli nrd*A Gene[a]

| residue[b] | isotope effect[c] | residue[b] | isotope effect[c] |
|---|---|---|---|
| C-25 | 1.0 (±0.2) | C-50 | 1.0 |
| C-29 | 1.0 | C-59 | 1.2 |
| C-32 | 1.4 | C-68 | 1.1 |
| C-42 | 1.1 | | |

[a] Reactions were carried out under normal atmospheric conditions as described under Materials and Methods. [b] Numbered residues are located in the following sequence: 5′...CGC$_{25}$AAGC$_{29}$AGC$_{32}$GTGTGAAAG-C$_{42}$CGTTGAGC$_{50}$TGTTCTCGC$_{59}$TGATGATGC$_{68}$AGGA...3′. [c] Isotope effects are for the total cleavage (P + PG) at a given site.

*Modulation of Isotope Effects.* The variability of isotope effects observed for cleavage at dGT steps reinforces the hypothesis that the sequence context of the cleavage site can modulate the effect. This modulation could be due to changes in the rate of discriminatory processes ($k_X$) or in the structure and conformation of the transition state for hydrogen transfer (i.e., $k_H/k_D$) or both. That dGT steps can vary considerably in the extent of damage by BLM supports the importance of neighboring residues in the modulation of the physical and chemical steps. There does not, however, appear to be a correlation of the extent of damage at a specific site and the magnitude of the isotope effect.

Alternately, changes in the structure and conformation of the activated Fe·BLM complex itself could result in the modulation of the isotope effect at a specific sequence site for the same reasons—changes in the rates of discriminatory processes or the transition-state structure or both. This is clearly illustrated with tallysomycin A (TLM). TLM is a naturally occurring BLM analog (Kawaguchi et al., 1985) that has an antibiotic profile similar to that of BLM. In addition, TLM exhibits a similar DNA cleavage profile with specificity for dGC and dGT steps and generates both 3′-phosphate and 3′-phosphoglycolate termini. However, it is ~100 times less active than BLM in the cleavage of DNA with Fe(II) and $O_2$ (Kross et al., 1982). TLM contains, near the bithiazole moiety, the amino sugar 4-amino-4,6-dideoxy-L-talose. This amino sugar is not found in BLM and might be expected to have an effect on the binding affinity and structure of the activated complex and, therefore, the resulting isotope effects.

Fe·TLM exhibited a sequence specificity nearly identical to that of Fe·BLM, as previously reported (Kross et al., 1982). In addition, clear evidence for regiospecific 4′-hydrogen abstraction was observed (data not shown). The isotope effects on 4′-hydrogen abstraction by Fe·TLM are compared to those determined for Fe·BLM in Table III. The effects were smaller than those for Fe·BLM, ranging from 1.8–2.9 compared to 3.4–7.0 at the same T residues for Fe·BLM. We do not believe

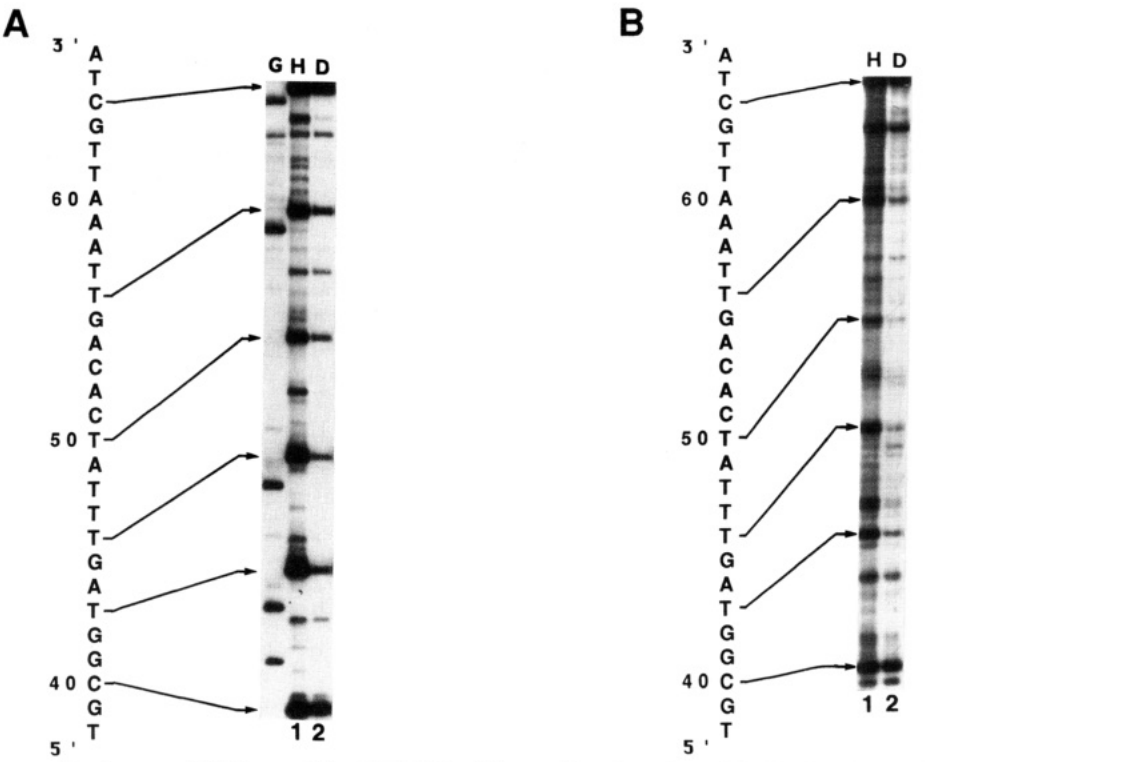



FIGURE 3: Isotope effects on the cleavage of DNA containing [4′-$^2$H]thymidine residues by activated Fe·BLM under high $O_2$ (A) and anaerobic conditions (B). (A) The 5′-$^{32}$P-end-labeled *Hin*dIII/*Bam*HI fragment of pBR322 containing thymidine (lane 1) or [4′-$^2$H]thymidine (lane 2) was cleaved by Fe(II) and BLM in 3 atm of $O_2$ as described under Materials and Methods followed by piperidine treatment, gel electrophoresis, and autoradiography. DNA fragments containing 3′-phosphoglycolate termini are the predominant products (pathway A, Scheme I). (B) The 5′-$^{32}$P-end-labeled *Hin*dIII/*Bam*HI fragment of pBR322 containing thymidine (lane 1) or [4′-$^2$H]thymidine (lane 2) was cleaved by Fe(III) and BLM with $H_2O_2$ as described under Materials and Methods followed by piperidine treatment, gel electrophoresis, and autoradiography. DNA fragments containing 3′-phosphate termini are the predominant products (pathway B, Scheme II).

Table III: Isotope Effects on 4′-Hydrogen Abstraction from Thymidine by Activated Fe·BLM and by Activated Fe·TLM on the *Hin*dIII/*Bam*HI Fragment of pBR322[a]

| | isotope effect[c] | |
|---|---|---|
| residue[b] | Fe·TLM | Fe·BLM |
| C-40 | 1.2 | 1.2 |
| T-43 | 2.3 | 4.2 |
| T-46 | 1.8 | 4.2 |
| T-48 | 2.9 | 6.4 |
| T-50 | 2.3 | 3.6 |
| T-56 | 2.5 | 3.5 |

[a] Reactions were carried out under normal atmospheric conditions as described under Materials and Methods. [b] Residues of the (+) strand of pBR322 are numbered from the *Eco*R1 site. [c] Isotope effects are for the total cleavage (P + PG) at a given site.

that the smaller magnitude of the isotope effects observed for Fe·TLM is related to the decreased extent of damage. The higher concentrations of Fe·TLM required for cleavage may be interpreted as due to poorer binding which may be explained, in part, by an increase in $k_X$ (eq 1). In lieu of other factors, this would lead to larger not smaller effects. These observations demonstrate that both structural alterations of the DNA or of the BLM can impinge on the parameters modulating the isotope effect without compromising the regiospecificity of hydrogen abstraction.

Replacement of Fe with another metal can lead to more significant changes in the cleavage mechanism and, hence, modulation of isotope effects. The photochemically induced cleavage of DNA by Co·BLM is an excellent example (Figure 4). The sequence specificity of cleavage is similar to that observed for Fe·BLM; however, fragments terminating in 3′-phosphoglycolate are not generally observed consistent with previous observations (Giloni et al., 1981). A subsequent report observing 3′-phosphoglycolate ends (Change & Meares, 1984) has not been corroborated by our findings or the work of others (Saito et al., 1989). Prior to treatment of the damaged DNA with piperidine, a predominance of ALM fragments are observed (lanes 1–3). The ALM fragments are cleanly converted to 3′-phosphate terminating fragments after treatment with piperidine (lanes 4–6). A careful inspection of an ALM fragment of relatively short length, such as C-40 or T-43, reveals that they resolve into doublets. We believe that the minor, slower-moving band is due to photoisomerization of the trans-ALM to the cis isomer. A similar observation has been made for the trans product generated by $\beta$-elimination from aldehydic abasic sites in DNA (Mazumder et al., 1991). Photoisomerization is supported by the observation that the ALM fragments generated by Fe·BLM will yield doublets when subsequently irradiated (data not shown). These findings are in agreement with recent studies with the Co(III)·BLM "green" complex (Saito et al., 1989) suggesting that C-4′ hydroxylation (Scheme I, pathway B) is the major pathway for deoxyribose fragmentation. However, we observe no significant isotope effect at any of the susceptible thymidine residues upon either 4′- or 5′-deuteriation (Figure 4).

A recent study by Mascharak and co-workers (Tan et al., 1992), using a synthetic ligand based on a minimal structure for the metal-binding domain of BLM, has led to an interesting proposal for the oxygen-independent, light-induced cleavage of DNA by Co-BLM. They have suggested that, upon UV irradiation of the Co·ligand complex, an unstable ligand radical is generated which is capable of producing hydoxyl radical from $H_2O$. Generation of hydroxyl radical in close proximity to the DNA would result in DNA cleavage. Spin trapping experiments have resulted in the detection of hydroxyl radical

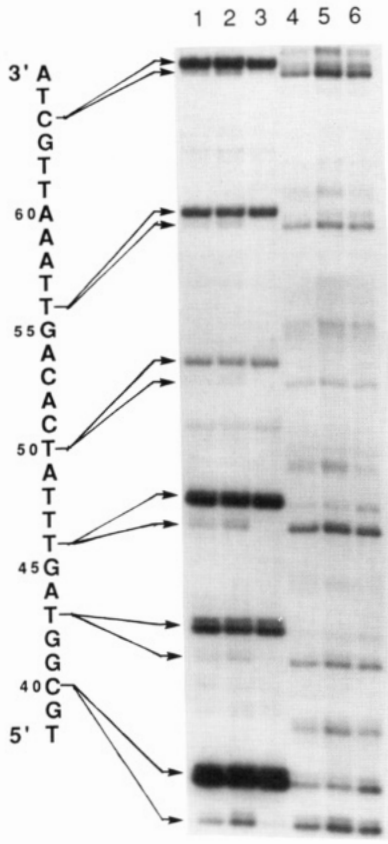



FIGURE 4: Isotope effects on the photochemically induced cleavage of DNA containing [4′-$^2$H]- or [5′,5′-$^2H_2$]thymidine residues by Co·BLM. The 5′-$^{32}$P-end-labeled *Hin*dIII/*Bam*HI fragment of pBR322 containing thymidine (lanes 1 and 4), [4′-$^2$H]thymidine (lanes 2 and 5), or [5′,5′-$^2H_2$]thymidine (lanes 3 and 6) was cleaved by Fe(II) and BLM as described under Materials and Methods, separated by electrophoresis on a 20% denaturing polyacrylamide gel, and subjected to autoradiography. Samples were analyzed prior to (lanes 1–3) and after (lanes 4–6) piperidine treatment.

although in very low amounts relative to the concentration of the Co·ligand complex. Their inability to suppress DNA cleavage with radical scavengers was explained by the proximity effect which permits trapping of only those hydroxyl radicals that have diffused away from the DNA (Tan et al., 1992). While our findings do not exclude this possibility, the regiospecificity for 4′-hydrogen abstraction renders this proposal problematic since, in general, hydroxyl radical lacks this specificity (vide infra). The low yields of hydroxyl radical detected by these researchers are also consistent with its formation as a side reaction from the activated complex which is responsible for regiospecific DNA cleavage. Since DNA cleavage in this study was measured by $\phi$X174 (RF) DNA nicking, the chemistry of DNA cleavage by these complexes cannot be assessed. Therefore, the relevance to Co·BLM is not clear.

An earlier analysis of the sites of binding and of cleavage of DNA by Fe·BLM and Co·BLM has also led to the proposal that the observed differences are the result of a change in mechanism of strand cleavage which may be due, in part, to the production of a highly reactive and diffusable species (McLean et al., 1989). Damage created by this species was observed most clearly at dAA and TT sites in homopolymeric tracts of up to six base pairs. Co·BLM was found not to bind to these regions on the basis of footprinting experiments, but attempts to suppress damage with radical scavengers at these remote sites were unsuccessful. In general, however, the sequence selectivities for Fe·BLM and Co·BLM were found to be quite similar for dGT and, to a lesser extent, dGC sites, and the observation of differences was clearly dependent upon the DNA sequence studied. Our findings are consistent with the observed similarities of the two BLM complexes, and our DNA sequence may not be appropriate for the study of dAA and TT sites where differences would be most apparent.

The two studies cited above suggest that hydroxyl radical or an equivalent species generated may play a role in DNA damage by photoactivated Co·BLM. However, the high sequence specificity and high regiospecificity of DNA cleavage and the inability to trap or detect significant levels of diffusable radicals suggest that the role is a minor one. Nevertheless, our findings are consistent with a fundamental difference between Co·BLM and Fe·BLM in the kinetics, mechanism, and reactivity of DNA cleavage that these two studies demonstrate (Tan et al., 1992; McLean et al., 1989).

*Conclusions.* The homolytic cleavage of a carbon–hydrogen bond in the deoxyribose moiety of a nucleotide residue in DNA is one of the major pathways leading to DNA damage that is available to a variety radical-generating reagents and drugs. Recent findings with other cleavage systems suggest that the isotope effects observed with BLM may be an exception rather than the rule. In the case of hydroxyl radical or its equivalent, which can be produced by ionizing radiation or Fenton-type chemistry, the reaction shows no sequence specificity and affords products consistent with multiple sites of carbon–hydrogen bond homolysis (Beesk et al., 1979; Tullius & Dombroski, 1985). The high diffusibility and reactivity of the hydroxyl radical species is consistent with these observations. Studies with Fe·EDTA (T. Tullius, personal communication) have revealed, at best, small effects ($k_H/k_D$ = 1.1–1.2) on specific hydrogen abstraction. This is certainly due to the low specificity and high reactivity of the putative hydroxyl radical species. Sequence specificity with Fe·EDTA can be gained by tethering the chelate to a sequence recognition element, such as the minor groove binders netropsin and distamycin (Dervan, 1986). In these cases, the observation of "tattered ends" suggests a localized production of a diffusible and reactive radical. However, isotope effects remain small, suggesting that while some sequence specificity has been achieved, regiospecificity of hydrogen abstraction has not.

Regiospecificity of carbon–hydrogen bond cleavage, of course, does not guarantee an isotope effect. High sequence and chemical specificity has been observed for a number of compounds that generate intrinsic oxygen- or carbon-based radicals which are highly localized and not subject to significant diffusion. Thus, the 1,10-phenanthroline–copper complex (Sigman, 1986), neocarzinostatin (Goldberg, 1991), the esperamicins (Christner et al., 1992), and the calicheamicins (Lee et al., 1991), enediyne antibiotics, have been shown to effect carbon–hydrogen bond homolyses consistent with placement of the substrate-bound radical in the minor groove (i.e., 1′-, 4′-, or 5′-C–H bond cleavage). In the case of the enediyne and related antibiotics, the record is mixed but has been informative. Isotope effects on 1′-hydrogen abstraction from dC in dAGC steps (Kappen et al., 1990) and on the kinetic wobble effect of 4′- and 5′-hydrogen abstraction from T in dGT steps (Frank et al., 1991; Kappen et al., 1991) have been valuable in assessing the chemistry of cleavage at minor damage sites. However, no significant isotope effects have, thus far, been detected with the esperamicins (Kozarich et al., 1989; Christner et al., 1992).

Fe·BLM is, therefore, a special case. High regioselectivity for and a low commitment to hydrogen abstraction and favorable parameters of transition-state orientation and

symmetry have created optimal conditions for the direct observation of deuterium selection effects at individual sequence sites. The effects are large and sensitive to sequence environment of the cleavage site and to structural and electronic changes in the metal·BLM complex. The power of the methodology is apparent and should prove invaluable for such studies as the detection of remote, interstrand isotope effects potentially associated with a proposed model for the double-stranded cleavage of DNA by Fe·BLM (Povirk et al., 1989; Steighner & Povirk, 1990). These studies are in progress.

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