species eluting at 23 min is 3-*O*-[5'-(2-deoxyadenylyl)]-2-deoxypentitol. Similar limiting O_2 experiments were carried out with sonicated calf thymus DNA and poly(dG-dC). In both cases [1,4-$^2H_2$]-2-deoxypentitols were identified.

Quantitation of 3a-c and Correlation to Base Release. To circumvent any uncertainties associated with differential recovery of monomeric and oligomeric products through multiple chromatographic procedures, a simplified degradation procedure was used for quantitative analysis. Subsequent to treatment of DNA (polymer or oligomer) with activated BLM, the lesion was reduced with $NaBH_4$ and digested directly with P_1 nuclease/alkaline phosphatase. HPLC analysis then allowed a direct comparison to be made between uracil and **3c** (Figure 3) in the case of poly(dA-dU) and cytosine and **3a** and **3b** in the case of d(CGCGCG) and poly(dG-dC) (Figure 3). The results are summarized in Table III. For d(CGCGCG), quantitative recovery (99%) compared to cytosine release occurs under these conditions. With poly(dA-dU), 81% of the uracil released could be accounted for by isolation of **3c**. The recovery of **3b** relative to cytosine release from poly(dC-dG) was only 48%. The poor quantitation with poly(dG-dC) may reflect difficulty associated with the synthesis and hence heterogeneity of this polymer.[39] Such factors could influence the stability of the lesion toward elimination prior to trapping with $NaBH_4$.

The results in Table III establish that $NaBH_4$ trapping is efficient compared to elimination across the C-2'-C-3' bond and prove that formation of a 4'-ketone accounts for the majority of the alkaline-labile sites produced by Fe-BLM.

Conclusions

The results reported here firmly establish the identity of the alkaline-labile lesion in the BLM reaction as a 4'-keto-1'-aldehyde. Our findings constitute the first verification of a near stoichiometric production of this lesion with respect to nucleic acid base release. Moreover, the generality of the analysis is demonstrated by our studies on a variety of heterogeneous DNAs.

A different approach to identification of the alkaline-labile lesion has recently been reported by Sugiyama et al.[13,14] The method involves strongly basic conditions to effect the rearrangement of the lesion and has not been quantitated with respect to base release. Our attempts to utilize this approach with **1a** (Figure 3) resulted in a mixture of products intractable to identification and quantitation. The results obtained by their procedures may therefore constitute a specialized case lacking the generality of the procedures reported here.

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Identification of the Source of Oxygen in the Alkaline-Labile Product Accompanying Cytosine Release during Bleomycin-Mediated Oxidative Degradation of d(CGCGCG)

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Abstract: Interaction of bleomycin, Fe^{2+}, and limiting O_2 or bleomycin, Fe^{3+}, and H_2O_2 with the hexamer d(CGCGCG) results in the production of an oxidatively damaged sugar lesion, 2'-deoxy-4'-pentulose, that is produced stoichiometrically with respect to free nucleic acid base release (Rabow et al., *J. Am. Chem. Soc.*, preceding paper in this issue). Similar studies using either ^{18}O-labeled O_2 or H_2O have established that the source of oxygen in the 4'-keto moiety of the 2'-deoxy-4'-pentulose is the solvent. The implications of these results with respect to the mechanism of nucleic acid base release in bleomycin-mediated DNA degradation are presented.

Bleomycin, an antitumor antibiotic,[1] in the presence of the required cofactors Fe^{2+}, O_2, and reductant or Fe^{3+} and hydroperoxide oxidatively degrades DNA to produce two types of lesions in a ratio of approximately 1:1.[2-4] This ratio can be modulated by the O_2 concentration.[5] Under anaerobic conditions, the predominant lesion results in the release of free nucleic acid base, which is accompanied by strand scission upon subsequent treatment with hydroxide. At elevated O_2 concentrations, the predominant lesion results in the production of base propenal, which is accompanied by strand scission under neutral conditions to produce 3'-phosphoglycolate and 5'-phosphate ends.[2-5]

Previous studies from our laboratories[6,7] using 4'-^3H-labeled DNA models established that both types of lesions were the result of labilization of the 4'-carbon-hydrogen bond of a pyrimidine

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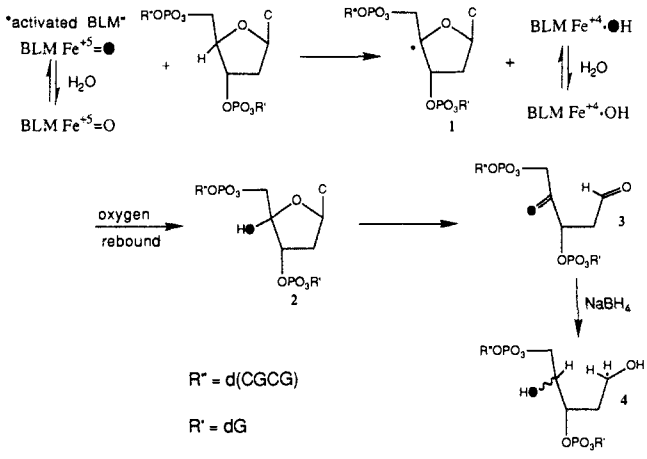

Figure 1. Proposed mechanism for the formation of the oxidized sugar produced concomitant with base release in the degradation of DNA by activated BLM.

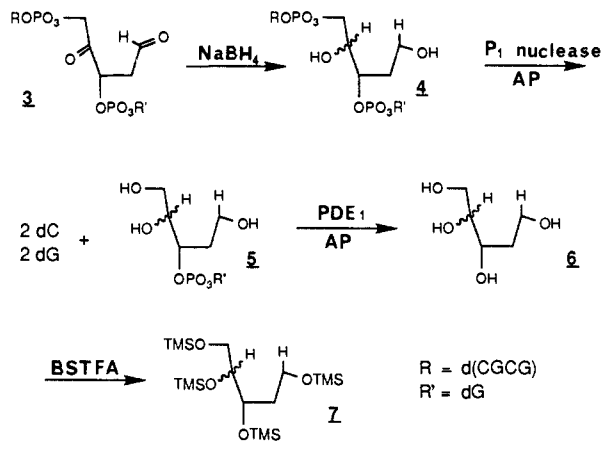

Figure 2. Protocol for the analysis of the ^{18}O content of the alkaline-labile product accompanying base release in the BLM-mediated DNA degradation reaction.

moiety in a purine–pyrimidine sequence, resulting in the production of a common intermediate whose fate was dependent on the O_2 concentration. Recently these studies have been extended to DNA fragments containing [4′-^{2}H]thymidine residues. Primary kinetic isotope effects have been observed at individual thymidine residues by using DNA sequencing technology on both types of DNA damage.[24] The chemistry observed in the model systems appears to be very similar to that observed with DNA.

As documented in detail in the previous paper in this series, production of free nucleic acid base is accompanied by the *stoichiometric* production of carbohydrate moiety **3** (Figure 1) regardless of the source of activated BLM or the source of DNA (oligonucleotides, copolymers, or calf thymus DNA).[8] A model to accommodate these observations is presented in Figure 1.

A mechanism for the production of these products is based on the extensive literature involving heme model compounds,[9] which suggests that activated BLM, a putative Fe^{3+} hydroperoxide, can undergo degradation via heterolytic oxygen–oxygen bond cleavage to produce an iron oxene ($Fe^{3+}O \leftrightarrow Fe^{5+}{=}O$) that rapidly abstracts a hydrogen atom from the C-4′ position of a deoxypyrimidine moiety of DNA to yield a 4′-radical (**1**). (*Nothing is known about the structure of the putative activated BLM subsequent to oxygen–oxygen bond cleavage. The nomenclature we have chosen to use has no structural implications.*) Recombination of the hydroxyl radical equivalent and the 4′-carbon radical results in the production of **2**, which then could collapse to release base and **3**. This model predicts that the oxygen in the "C-4′" position of **3** is derived from "O_2" required to produce activated BLM. Results of studies to test this prediction are presented in this paper. In contrast to the predicted outcome (Figure 1), the C-4′ oxygen is shown to be derived almost exclusively from H_2O. A modified mechanism is presented to accommodate these unexpected results.

Experimental Section

Materials. The hexanucleotide d(CGCGCG) was a kind gift of John Gerlt at the University of Maryland. Blenoxane, a clinical bleomycin (BLM) mixture containing 60% BLM A_2 and 30% BLM B_2, was a gift of Bristol-Myers. P_1 nuclease and snake venom phosphodiesterase (PDE I) were purchased from Pharmacia. One unit of P_1 nuclease hydrolyzes 1 μmol of 3′-AMP min^{-1} at 37 °C and pH 7.2. and 1 unit of PDE I hydrolyzes 1 μmol of *p*-nitrophenyl phosphate min^{-1} at 25 °C and pH 8.9. *Escherichia coli* alkaline phosphatase (1 unit hydrolyzes 1 μmol of *p*-nitrophenyl phosphate min^{-1} at 37 °C and pH 10.4), deoxynucleosides, DNA bases, $NaBH_4$, and NaB^2H_4 (98 atom %) were obtained from Sigma. 2-Deoxy-D-ribose was purchased from Aldrich. $H_2{}^{18}O$ (95 atom %) was supplied by Amersham, and $^{18}O_2$ (95 atom %) was a product of MSD Isotopes. *N,O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) and dry acetonitrile used as silylation cosolvents were purchased from Pierce. C_{18} reverse-phase HPLC columns were obtained from Alltech (10 μm) or Beckman (5 μm). All other reagents were of the highest available laboratory grade.

HPLC analyses were performed by using a Beckman system with Model 110A pumps. Detection was by UV absorption at 254 or 260 nm. ^{1}H NMR spectra were obtained by using a Brüker 250-MHz spectrometer, and ^{13}C NMR spectra were taken on a Varian VXR 500-MHz spectrometer. The glassware used for samples analyzed by mass spectrometry was silanized prior to use with 5% dimethyldichlorosilane in $CHCl_3$. After trimethylsilyl (TMS) derivatization, samples were analyzed by GC/MS using a Kratos MS 25 system with a 10-m OV-17 capillary column (Alltech) or a 30-m homemade equivalent. Alternatively, a Hewlett-Packard 5890 GC/5970A MS with a 30-m DB-5 capillary column was used. Detection in both cases used total ion current. UV/visible spectra were recorded on a Cary 210 or Beckman DU-50 instrument.

Identification of the Source of Oxygen in C-4′ of 3. (A) BLM–$^{18}O_2$–Fe^{2+}. Reaction mixtures contained, in a final volume of 500 μL, 0.21 mM BLM, 0.84 mM d(CGCGCG) (in nucleotides), 0.42 mM $^{18}O_2$ (95 atom %), and either 40 mM triethanolamine (pH 7.8) and 100 mM $NaBH_4$ or 402 mM CHES (pH 9.0) and 60 mM $NaBH_4$. In a typical reaction, solutions of $Fe(NH_4)_2(SO_4)_2{\cdot}6H_2O$ in H_2O and $NaBH_4$ in 10 mM buffer at pH 10 were both purged with argon and added to a degassed solution containing the oligonucleotide and BLM. The reaction was initiated by adding $^{18}O_2$-saturated H_2O (1.3 mM $^{18}O_2$ at 25 °C[10]) with a gastight Hamiltonian syringe and was continued at 0 °C for 45 min. The reaction was then quenched with 20 μL of acetic acid. The reaction mixture was neutralized with NaOH and injected onto a C_{18} reverse-phase HPLC column, and **4** (Figure 1) was eluted with a 0–20% linear gradient over 10 min, flow rate 1 mL min^{-1}, in which buffer A contained 5 mM NH_4OAc (pH 5.5) and buffer B was CH_3OH. Compound **4** was enzymatically digested to give **6** and prepared for GC/MS analysis as previously described (Figure 2).[8]

(B) BLM–$^{16}O_2$–Fe^{2+}–$H_2{}^{18}O$. The experimental conditions were identical with those described above except that the reaction mixture contained 50% $H_2{}^{18}O$. After the reaction mixture was quenched with acetic acid (pH 5.0), P_1 nuclease (5 units) was added and the mixture was incubated for 1.5 h at 37 °C. The mixture was frozen and the H_2O recovered by bulb to bulb distillation. Determination of the $H_2{}^{18}O$ content of the distillate will be described subsequently. The residue was dissolved in 400 μL of 56 mM Tris-HCl (pH 7.5) containing 3 units of alkaline phosphatase, and the mixture was incubated for 30 min at 37 °C. The products were isolated by C_{18} reverse-phase HPLC by isocratic elution with buffer A and 5% CH_3OH for 10 min, followed by a 5–20% linear gradient with CH_3OH over 15 min. Product, elution time (min), and recovery (nmol) were as follows: cytosine (C), 5.5, 17.3; 3-*O*-[5′-(2′-deoxyguanylyl)]-2-deoxypentitols (**5**), 8, 16.3; deoxycytidine (dC), 9.5, 136.1; deoxyguanosine (dG), 21.3, 133.7.

(C) Control Reactions: Exchange of C-4′ Oxygen of 3 with Solvent $H_2{}^{18}O$. Reactions under limiting oxygen in 500-μL total volume [triethanolamine (pH 7.8) or CHES (pH 9.0)] were identical with those described above except that $NaBH_4$ was omitted. After 45 min the

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reaction mixture was passed over a 0.6-mL Chelex column equilibrated with the appropriate buffer at 4 °C to remove the Fe–BLM. The column was washed with 500 μL of this buffer and the combined eluents were concentrated to a volume of 115 μL. $NaBH_4$ in 50 μL of triethanolamine (pH 7.8) or CHES (pH 10) was combined with $H_2{}^{18}O$ and added with rapid mixing to the reaction mixture containing lesion **3**. The final concentrations were 40 mM triethanolamine (pH 7.8) and 100 mM $NaBH_4$ or 40 mM CHES (pH 9.0) and 60 mM $NaBH_4$, identical with the conditions described above. The reaction was quenched after 45 min at 0 °C with 20 μL of acetic acid, the $H_2{}^{18}O$ was recovered to be analyzed as described subsequently, and the products were analyzed as described in section A. Product, elution time (min), and recovery (nmol) were as follows: C, 8, 28: **4**. 15, 21.2; d(CGCGCG), 17.5, 17.6. Compound **4** was isolated and digested with P_1 nuclease [5 units, 1.4 mM $ZnCl_2$ and 20 mM NaOAc (pH 5.5) for 1.5 h at 37 °C] and alkaline phosphatase [3 units, 56 mM Tris-HCl (pH 7.5) for 30 min at 37 °C]. Compound **5** was isolated by HPLC as described above. Product, elution time (min), and recovery (nmol) were as follows: **5**, 10, 18.7; dC, 11.5, 41.4; dG, 25, 43.0.

Measurement of the $H_2{}^{18}O$ Content from the Experiments Described in Sections B and C. H_2O (100 μL) isolated by bulb to bulb distillation in sections B and C was added to 1 mg of dried benzoic acid and 1 μL of 12 N HCl. The mixture was heated at 95 °C in a sealed reaction vial for 10 days. The H_2O was then removed and coevaporated with absolute ethanol, the sample dried over P_2O_5, and the benzoic acid derivatized with 30 μL of BSTFA (1% TMCS) by heating at 95 °C for 60 min. Controls showed that the exchange of ^{18}O was complete under these conditions. Samples were analyzed by using a Hewlett-Packard 5890 gas chromatograph 5970A mass spectrometer by injection onto a 30-m DB-5 column (J&W Scientific) and elution with a temperature gradient of 80–200 °C at 10 °C min^{-1}, followed by 200–300 °C at 30 °C min^{-1}. The TMS-benzoic acid derivative eluted at 7.2 min.

Determination of the ^{18}O Content of 4 by Mass Spectrometry. The 2-deoxypentitols **6** (5–20 nmol) were liberated from **5** by digestion with 3 units of PDE I in 10 mM $MgCl_2$ and 50 mM Tris-HCl (pH 7.5) for 1.5 h at 37 °C. The solution was deionized by passage over 400-μL columns of Dowex 50 (H^+) and Dowex-1-formate. The eluent was lyophilized and the residue transferred to a silanized reaction vessel by three successive washes with CH_3OH. The solution was evaporated to dryness, further dried with 200 μL of absolute ethanol, and then dried in vacuo over P_2O_5 for 12 h. The samples were derivatized with 10 μL of a 1:1 CH_3CN:BSTFA mixture (1% TMCS) by heating at 50 °C for 1 h. The TMS derivatives (**7**) of **6** were analyzed by GC/MS as described above; the two diastereomers (partially resolved) eluted at 12 min.

Mass Spectral Data Analysis. Peaks in the gas chromatogram were analyzed by using the Hewlett-Packard 1000 data system. The peak was summed by using the area sum method, where the peak maximum and the fronting and trailing background were specified. Nonisotopically labeled benzoic acid and 2-deoxypentitol standards were used to develop correction tables. Calculated percentages of the fragment (F) intensities were subtracted from the $F + 2$ and $F + 4$ intensities as correction factors. These corrected values were used to calculate the isotopic distribution.

The isotopic distribution in TMS-benzoate was determined from two clusters (105, 107 and 179, 181, 183). The appropriate equations for the fraction of ^{18}O are

$$107_{corr}/105 + 107_{corr}$$

and

$$[\tfrac{1}{2}(181_{corr}) + 183_{corr}]/(179 + 181_{corr} + 183_{corr})$$

For the 2-deoxypentitols, three fragments contained the C-1 oxygen but not the C-4 oxygen: 103, 219, and 307. These three fragments were averaged to determine isotopic distributions. Two fragments contained both the C-1 and C-4 oxygens: 231 and 321. The 205 fragment contained the C-4 oxygen only. The ^{18}O content at C-4 was determined by averaging the ^{18}O content determined at the 205, 231, and 321 fragments and subtracting two-thirds of the ^{18}O content at C-1. (Please note that the labeling convention here retains the numbering scheme for 2-deoxyribose and the 2′-deoxy-4′-pentulose product. C-1 refers to the aldehyde and C-4 refers to the ketone of the unreduced product **3**. Strictly speaking, the order of the numbering would reverse when this 1′-aldehyde 4′-ketone is converted into its sugar alcohol derivative.)

Synthesis of [1-$^2H_1$]-2-Deoxy-D-*erythro*-pentitol. [1-$^2H_1$]-2-Deoxy-D-*erythro*-pentitol was synthesized by using the procedure of Tymiak and Rinehart.[11] NaB^2H_4 (88.3 mg, 2.11 mmol, 5.7 equiv) was gradually added as a solid powder to 2-deoxy-D-ribose (200 mg, 1.49 mmol) in 12 mL of ethanol. The reaction was quenched after incubation at room temperature for 6 h by addition of acetic acid (apparent pH was 4.5). The solution was decationized by passage over a 5-mL Dowex-50-X8 (H^+) column in ethanol, the solvent was removed in vacuo, and borate was removed as methyl esters by repeated evaporations with methanol to yield essentially quantitative recovery of a pale yellow oil. ^1H NMR (Brüker 250 MHz, D_2O): δ 3.63–3.55 (3 H, m), 3.48–3.40 (2 H, m), 1.70 (1 H, ddd, J = 2.7, 7.75, 14.3 Hz), 1.49 (1 H, ddd, J = 5.45, 9.5, 14.6 Hz). ^{13}C NMR (125.7 Mz, D_2O, proton decoupled, dioxane as external standard at 66.5): δ 74.67 (C-4), 68.83 (C-3), 62.32 (C-5), 58.12 (triplet, J_{CD} = 21.6 Hz, C-1), 33.94 (C-2).

Table I. Incorporation of ^{18}O into C-1 and C-4 of TMS Deoxypentitols (**7**, Figure 2) during BLM-Mediated Degradation of d(CGCGCG)

source of label	% ^{18}O incorporation into **7**[a]	
	C-4	C-1
$^{18}O_2$, pH 7.8[b]	−2.7	−1.2
$^{18}O_2$, pH 9.0[b]	1.3	2.2
$H_2{}^{18}O$, pH 7.8[b,c]	97.8	5.4
$H_2{}^{18}O$, pH 9.0[b,d]	85.7	12.2

[a] The percent ^{18}O incorporated into C-4 and C-1 of **7** was established by analysis of its MS fragmentation patterns as described in the Experimental Section. [c] Fe^{2+}, BLM; and O_2 were incubated with d(CGCGCG) under the conditions described in the Experimental Section. [b] Based on 44.5% $H_2{}^{18}O$. [d] Based on 48.5% $H_2{}^{18}O$.

Figure 3. Postulated fragmentation pattern of the TMS derivative of the deoxypentitols **7** (Figure 2).[13]

Results

Interaction of BLM–Fe^{2+}–$^{18}O_2$ with d(CGCGCG): Analysis of 4. The mechanism proposed for the production of nucleic acid base by Fe^{2+}–BLM–O_2 (Figure 1) predicts that use of $^{18}O_2$ should result in the incorporation of "^{18}O" at the C-4′ of **3**. Previous studies from our laboratories indicated that oligomer d(CGCGCG) is an excellent model to investigate this hypothesis.[8,12] The oligomer was, therefore, incubated with Fe^{2+}–BLM and $^{18}O_2$ under a variety of pH and buffer conditions. The reduced deoxypentitols **6** were isolated subsequent to $NaBH_4$ reduction and enzymatic digestion and analyzed as previously described (Figure 2).[8] The results of mass spectral analyses are summarized in Table I and indicate that no ^{18}O is incorporated into the C-4 position at either pH 7.8 or pH 9.0.

A partial analysis of the mass spectral data that led to the calculation of the percent ^{18}O incorporation into C-4 and C-1 is as follows. The fragmentation patterns of the TMS deoxypentitols **7** were previously established and are shown in Figure 3.[13] If these fragmentation patterns are valid, then the fragments at m/z 205 and 307 contain only the C-4 oxygen, the fragments at 103 and 219 contain only the C-1 oxygen, and the fragment at 321 contains both C-1 and C-4 oxygens. As will be shown subsequently, the fragmentation patterns are correctly assigned except for 307. This fragment will be shown to contain only the C-1 oxygen and no C-4 oxygen. In addition, the fragment at 231 contains both the C-1 and C-4 oxygens. Analysis of these corrected patterns, described in the Experimental Section, led to the results in Table I.

Determination of the Source of Oxygen at C-4′ of 4. The results of the $^{18}O_2$ experiment can be accommodated by several alternative hypotheses (Figure 4). (1) The oxygen at C-4′ of **3** could be derived from $H_2{}^{18}O$ by an electron-transfer pathway described in Figure 4, path A. (2) Alternatively, the source of oxygen at

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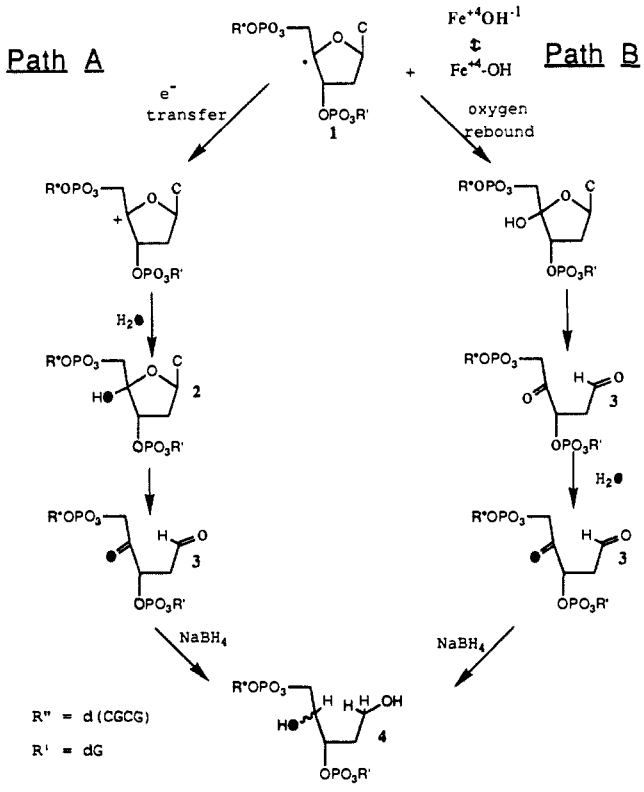

Figure 4. Alternative mechanisms to account for the observed labeling patterns with $^{18}O_2$ and $H_2{}^{18}O$ when d(CGCGCG) is incubated with activated BLM.

C-4′ could initially be $^{18}O_2$, but the rapid exchange of the 4′-ketone **3** with solvent would result in washout of this label (Figure 4, path B). The C-4′ oxygen of **3** would, therefore, also be derived from $H_2{}^{18}O$.

Experiments to determine if H_2O provides the source of oxygen at C-4′ were carried out, in which Fe^{2+}–BLM (limiting O_2), d(CGCGCG), and $NaBH_4$ were incubated in 40 mM triethanolamine buffer (pH 7.8) or CHES buffer (pH 9.0) in $H_2{}^{18}O$–$H_2{}^{16}O$ (~1:1). Analysis of the deoxypentitols isolated as described in Figure 2 revealed a near quantitative incorporation of ^{18}O into the C-4 position (86–98%) and very low incorporation of ^{18}O into the C-1′ aldehyde position (5–12%) (Table I). The mass spectral results of a typical experiment are shown in Figure 5a. The fragmentation patterns and analysis will be presented subsequently.

In order to test the efficiency of the $NaBH_4$ trapping under these various conditions, the following control was therefore run.[26] Compound **3** was prepared and isolated by HPLC. After volume reduction, **3** was added to a mixture of $H_2{}^{18}O$–$H_2{}^{16}O$ and $NaBH_4$, and the deoxypentitols were isolated and analyzed by GC/MS. The mass spectral result of this control is shown in Figure 5b. This spectrum differs substantially from that in Figure 5a; compare *m/z* fragments 205, 231, and 321. The differences are quantitatively displayed in Tables III and IV. A direct comparison between the results of the experiments and controls unambiguously establishes that greater than 90% of the oxygen at C-4′ is derived from $H_2{}^{18}O$ (Table II) and that the ^{18}O incorporation is *not* the result of a rapid exchange of the oxygen of the C-4′ ketone **3** with solvent after the drug-mediated event (Figure 4, path B). These experiments have an excellent internal control, the C-1′ aldehyde. Since aldehydes are in general more easily hydrated than ketones,[25] the extent of chemical ^{18}O incorporation into C-1′ would appear to constitute an upper limit for chemical exchange. That almost *no* $H_2{}^{18}O$ is found in the C-1 position further substantiates the efficiency of the trapping methods.

Analysis of the Fragmentation Pattern of Deoxypentitols: Interpretation of GC/MS Data. Analysis of the ^{18}O-labeling studies in Tables I–IV and Figure 5 depends critically upon interpretation of the fragmentation patterns for TMS deoxypentitols. Previous

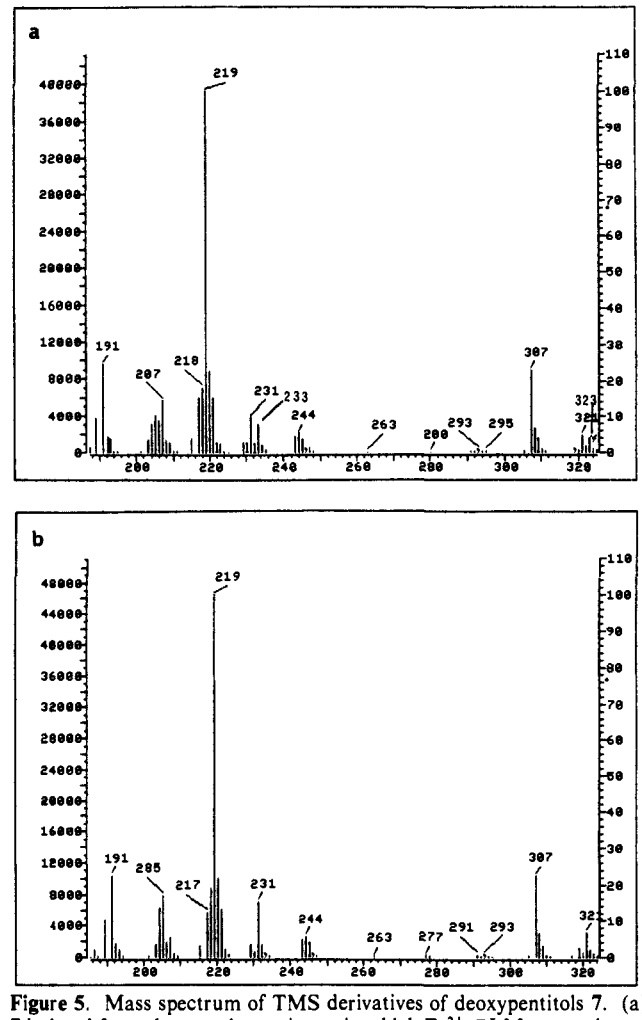

Figure 5. Mass spectrum of TMS derivatives of deoxypentitols **7**. (a) **7** isolated from the actual experiment in which Fe^{2+}–BLM was activated in the presence of 50% $H_2{}^{18}O$ and the product **3** (Figure 1) was trapped by $NaBH_4$ reduction. (b) **7** isolated from a control experiment in which **3** (Figure 1) was reduced with $NaBH_4$ in the presence of 50% $H_2{}^{18}O$.

Table II. Control Experiments Indicating the Efficiency of ^{18}O Trapping by $NaBH_4$

	% ^{18}O incorporation into **7**[a]	
pH	C-4	C-1
$H_2{}^{18}O$, pH 7.8[b,c]	3.8	0.2
$H_2{}^{18}O$, pH 9.0[b,d]	3.9	2.2

[a] The percent ^{18}O incorporated into C-4 and C-1 of **7** was established by analysis of its MS fragmentation patterns as described in the Experimental Section. [b] Compound **3** (Figure 1) was isolated as described in the Experimental Section and educed with $NaBH_4$ in the presence of $H_2{}^{18}O$. [c] Based on 45.3% $H_2{}^{18}O$. [d] Based on 50.1% $H_2{}^{18}O$.

Table III. $H_2{}^{18}O$ Incorporation into **7** at pH 7.8: Analysis of MS Fragmentation Patterns

BLM experiment[a]		control[b]	BLM experiment[a]		control[b]
fragment	ratio $^{16}O/^{18}O$	ratio $^{16}O/^{18}O$	fragment	ratio $^{16}O/^{18}O$	ratio $^{16}O/^{18}O$
205	47.7	95.5	103	97.5	99.6
207	52.3	4.5	105	2.5	0.4
231	58.5	100.3	219	98.4	100.2
233	41.5	−0.3	221	1.6	−0.2
321	58.5	98.7	307	96.8	99.9
323	41.5	1.3	309	3.2	0.1

[a] The BLM experiment (B) was run as described in the Experimental Section in 40 mM triethanolamine (pH 7.8), and the $H_2{}^{16}O$:$H_2{}^{18}O$ ratio was established to be 55.5:44.5 (Experimental Section). [b] The control experiment (C) was run as described in the Experimental Section, and the $H_2{}^{16}O$:$H_2{}^{18}O$ ratio was established to be 54.7:45.3 (Experimental Section).

Table IV. $H_2{}^{18}O$ Incorporation into **7** at pH 9.0: Analysis of MS Fragmentation Patterns

BLM experiment[a]		control[b]	BLM experiment[a]		control[b]
fragment	ratio $^{16}O/^{18}O$	ratio $^{16}O/^{18}O$	fragment	ratio $^{16}O/^{18}O$	ratio $^{16}O/^{18}O$
205	49.4	94.0	103	94.0	98.9
207	50.6	6.0	105	6.0	1.1
231	57.4	99.9	219	95.2	99.1
233	42.6	0.1	221	4.8	0.9
321	56.8	98.0	307	93.2	98.6
323	43.2	2.0	309	6.8	1.4

[a] The BLM experiment (B) was run as described in the Experimental Section in CHES (pH 9.0), and the $H_2{}^{16}O$:$H_2{}^{18}O$ ratio was established to be 51.5:48.5 (Experimental Section). [b] The control experiment (C) was run as described in the Experimental Section, and the $H_2{}^{16}O$:$H_2{}^{18}O$ ratio was established to be 49.9:50.1 (Experimental Section).

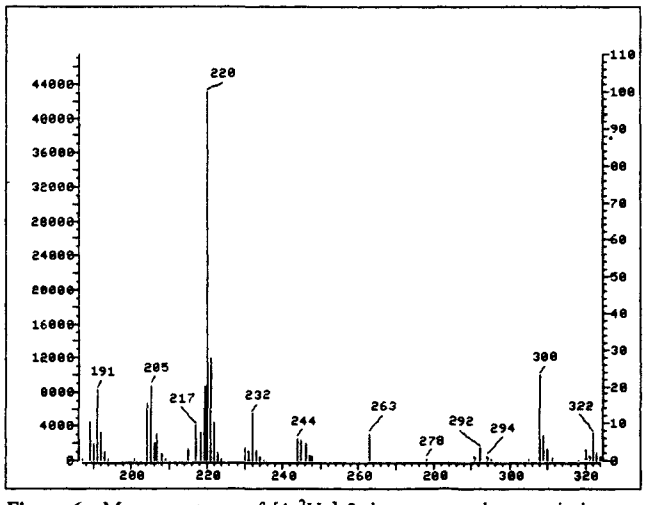

Figure 6. Mass spectrum of [1-$^2H_1$]-2-deoxy-D-*erythro*-pentitol.

studies of Beesk et al. had indicated that derivatized deoxypentitols fragmented as described in Figure 3.[13] These assignments were made by isotopic labeling studies using a variety of related pentitols derived from DNA damaged by ionizing radiation. Since the *m/z* 307 fragment has been postulated to contain the C-4 oxygen, the lack of a 309 fragment in the $H_2{}^{18}O$ experiment (Figure 5a) was particularly bothersome. Therefore, to establish the correct identity of the fragmentation patterns, [1-$^2H_1$]-2-deoxy-D-*erythro*-pentitol was prepared and examined. The results are shown in Figure 6. The most obvious discrepancy in the mass spectrum of the deuteriated deoxypentitol is the presence of a major *m/z* 308 and not the expected 307 (Figure 3), indicating the presence of deuterium at C-1.[13] An alternative fragmentation pattern must, therefore, be operative. We postulate that the *m/z* 307 arises from a hydrogen abstraction followed by a rearrangement as indicated in Figure 7. This type of fragment (M − 117) has been previously observed in the mass spectra of other alditol derivatives.[14] This rearrangement would now predict that the peak at 307 should not be enriched with ^{18}O as it contains no C-4 oxygen. The observed fragmentation pattern of [1-$^2H_1$]-2-deoxy-D-*erythro*-pentitol and the deoxypentitols derived from the $H_2{}^{18}O$-labeling experiment and control (Figure 5) are entirely consistent with this interpretation.

Discussion

When activated BLM degrades DNA, the alkaline-labile product that forms concomitant with base release results from a two-electron oxidation of the deoxyribose moiety. The most frequent damage site is a pyrimidine that is located on the 3′ side of a purine in DNA.[15,16] The studies reported here have used d(CGCGCG) as a model DNA substrate to investigate the source of oxygen incorporated into the alkaline-labile product **3** (Figures 1 and 4). This oligonucleotide has been previously shown to have a well-defined reproducible interaction with activated BLM.[8,12] At a 4:1 molar ratio of DNA to drug, the major site of attack is the penultimate deoxycytidine residue. Under standard atmospheric conditions, the d(CGCG) glycolate and **3** are produced in approximately a 1:1 ratio. Under conditions of limiting O_2, **3** is the major modified oligonucleotide produced.[8] Previous studies have shown that **3** can be nonstereospecifically reduced by $NaBH_4$ to yield **4**, the epimeric pair of 2-deoxypentitols that, subsequent to enzymatic degradation, can be analyzed by GC/MS as the TMS derivatives (**7**) (Figure 2). Thus d(CGCGCG) is an excellent model for the study of the mechanism of oxygen incorporation into the alkaline-labile sugar moiety.

Experiments with Fe^{2+}–BLM and limiting amounts of $^{18}O_2$ were conducted in $H_2{}^{16}O$–buffer systems in the presence of $NaBH_4$. These experiments, carried out at a variety of pH values, failed to show any evidence of ^{18}O incorporation in the isolated deoxypentitols. From these experiments, one can conclude either that the oxygens in **3** are not derived from molecular oxygen as predicted from our original hypothesis (Figure 1) or that solvent exchange from the 4′-ketone is much faster than $NaBH_4$ trapping (Figure 4, path B).

Control experiments (Tables II–IV) were conducted to test this latter point. Compound **3** was incubated with $H_2{}^{18}O$ and $NaBH_4$ under a variety of conditions. Only a small amount of label was incorporated in triethanolamine (pH 7.8) [C-4 (4%) and C-1 (0.2%)] and in CHES (pH 9.0) [C-4 (4%) and C-1 (2%)]. A strikingly different pattern was obtained when **3** was generated by Fe^{2+}–BLM in the presence of $NaBH_4$ and $H_2{}^{18}O$. There was a low level of incorporation of solvent oxygen at C-1 (5% and 12% in triethanolamine and CHES, respectively), but essentially all of the oxygen at C-4 was derived from solvent [98% and 86% in triethanolamine and CHES, respectively (Table I)]. This confirms the previous conclusion from the BLM–Fe^{2+}–$^{18}O_2$ experiments that solvent is the major source of the oxygen at C-4′ and demonstrates unambiguously that $NaBH_4$ trapping is rapid compared to exchange.

As indicated in Figure 1, by analogy with various heme model systems[17] and with cytochrome P_{450} and peroxidases,[9,18] Burger et al. suggested that the activated form of BLM might be an iron oxene ($Fe^{3+}O \leftrightarrow Fe^{5+}{=}O$).[2,19] Hydrogen atom abstraction at C-4′ would produce an $Fe^{4+}OH^- \leftrightarrow Fe^{4+}$–OH capable of undergoing a "rebound" mechanism (radical recombination) with the carbon-centered radical at C-4′ (**1**) (Figure 1). The above results establish that this is not a viable mechanism to produce compound **2** unless the $Fe^{3+}O$ or $Fe^{4+}OH^-$ is capable of undergoing rapid solvent exchange prior to rebound (Figure 1). While this possibility cannot be unambiguously excluded, several pieces of available evidence suggest that rapid exchange from the $Fe^{3+}O$ does not occur. First, model studies by Hecht and co-workers in which Fe^{2+}, BLM, ascorbate, and $^{18}O_2$ were incubated with *cis*-stilbene in aqueous CH_3OH resulted in the production of 90%-enriched ^{18}O-labeled *cis*-stilbene oxide.[20] If the ^{18}O is in fact derived from "activated BLM" and not subsequent O_2-mediated chemistry, than exchange does not appear to be rapid. However, it remains to be established whether the same activated Fe–BLM species is responsible for epoxidation and hydroxylation.

Second, EPR studies of Burger et al. using Fe^{2+}–BLM and $^{17}O_2$ indicate that activated BLM contains at least one oxygen derived from O_2.[2] These studies were not designed to measure exchange rates but indicate that in activated BLM exchange is slow. These

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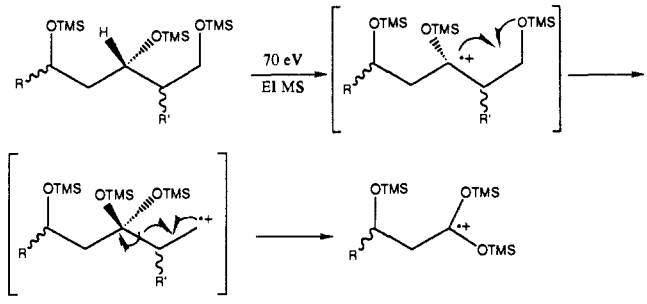

R =	R' =	m/z
H	^{16}OTMS	307
H	^{18}OTMS	307
D	^{16}OTMS	308

Figure 7. Postulated rearrangement accompanying fragmentation of **7** (Figure 2) that accounts for the observed ^{18}O- and ^{2}H-labeling patterns (Figures 5 and 6).

results do not preclude the possibility, however, that activated BLM observed by Burger et al. is the precursor to the real activated BLM produced by oxygen–oxygen bond scission and that the resulting monooxygenated Fe species ($Fe^{3+}O$ or $Fe^{4+}OH^-$; Figure 1) can undergo exchange. The washout of oxygen label from an iron–oxo species thus requires further investigation.

An alternative explanation for the observed incorporation of H_2O into C-4′ of **3** is provided in Figure 4, path A. The C-4′-centered radical **1** generated by hydrogen atom abstraction by the $Fe^{3+}O$ could be oxidized by electron transfer to the $Fe^{4+}OH^-$ to generate the C-4′ oxonium ion and $Fe^{3+}H_2O$. Addition of H_2O from solvent to the oxonium ion would produce the observed results. This mechanism retains the oxidation state of activated BLM originally proposed by Burger et al.[19] but replaces radical recombination (rebound) with electron transfer in the subsequent transformation. This mechanism is distinct from explanations involving solvent exchange, since incorporation of solvent oxygen is a mandatory part of this scheme.

Recent studies of Padbury et al. investigating BLM activated by 10-hydroperoxy-8,12-octadecadienoic acid require the consideration of yet another possibility, also involving oxonium ion production via an electron-transfer mechanism.[21] They were using this hydroperoxide as a tool to establish the mechanism by which the $Fe^{3+}(O_2R)^-$ is converted to an "FeO" species, i.e., whether O–O bond cleavage occurs by a homolytic or a heterolytic mechanism. Their results clearly demonstrate that with this hydroperoxide homolytic cleavage occurs under a variety of conditions, and their preliminary results monitoring the production of linear and nicked DNA from supercoiled DNA indicate that this type of cleavage supports DNA degradation. No direct correlation between DNA cleavages and homolysis, however, was presented. If BLM is in fact activated by homolytic oxygen–oxygen bond scission as these results suggest, then an alternative model analogous to the one described in Figure 4 (path A) can be put forth to accommodate the ^{18}O-labeling experiments. The $Fe^{3+}O_2^{2-}$ would be converted to HO• and $Fe^{3+}O^-$. The latter species would presumably be responsible for hydrogen atom abstraction from C-4′ to give a C-4′ radical and $Fe^{3+}OH^- \leftrightarrow Fe^{3+}$–OH. Oxidation of C-4′ by electron transfer to $Fe^{3+}OH^-$ would result in a C-4′ oxonium ion, which could react with H_2O to produce **3** and $Fe^{2+}OH^-$. This model predicts that Fe^{2+}–BLM should be detectable spectroscopically, as should the production of 1 equiv of HO•. Studies of previous investigators[22] utilizing spin traps have revealed the production of HO•, but the quantitation showed substoichiometric amounts (6% of the HO• produced with Fenton's reagent). However, no evidence for Fe^{2+}

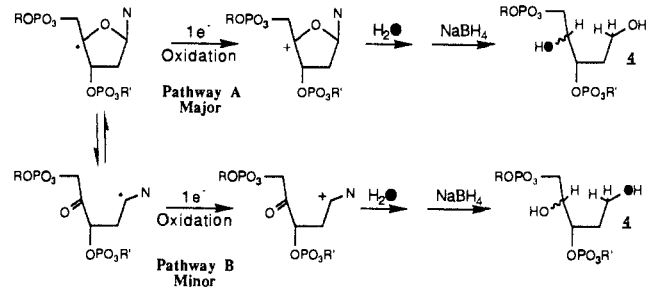

Figure 8. Postulated mechanism to account for the increased incorporation of ^{18}O at C-1 of **7** in the experiment (Table I) relative to the control (Table II).

production, easily detectable spectrophotometrically, presently exists (Rabow, unpublished observations). Alternatively, a second molecule of $Fe^{3+}O^-$ could catalyze this oxidation of the C-4′ radical **1** (Figure 4, path A), which would result in the production of the same C-4′ oxonium ion and Fe^{3+} (Fe^{3+}, H_2O) observed experimentally. If this latter model is correct, then efforts should be made to quantitate HO• produced. Fe^{3+} is the observed product of the reaction.[2]

At present, therefore, we cannot distinguish between the heterolytic mechanism shown in Figure 4 (path A) and the homolytic one that would require a second activated BLM to catalyze the same oxidation. Studies utilizing d(CGCGCG) and 10-hydroperoxy-8,12-octadecadienoic acid to generate activated BLM should allow a distinction to be made between these options. Furthermore, additional experiments will be required to rule out the possibility of exchange with solvent of an FeO species to account for the observed labeling results.

Finally, a comment is required on the results from the $H_2{}^{18}O$ experiment (Table I) in comparison with those from the $H_2{}^{18}O$ control experiment (Table II) conducted at pH 9.0. The control indicates that 2% of the solvent isotope can exchange into C-1′ prior to trapping by $NaBH_4$, while the experiment indicates that ~12% ^{18}O was found in C-1′, apparently at the expense of $H_2{}^{18}O$ in C-4′ (86%). A mechanism to accommodate these observations is indicated in Figure 8. Subsequent to hydrogen atom abstraction at C-4′ to generate a C-4′ radical **1**, this intermediate can partition to produce the same product **4** by two alternative pathways, A and B.

In the minor pathway B, a C-1′ radical is generated from the C-4′ radical **1** that can subsequently undergo oxidation and H_2O addition. In this case the C-1′ would contain ^{18}O from $H_2{}^{18}O$, and the C-4′ would contain oxygen derived from the C-4′ oxygen of the sugar. In the major pathway A, the major source of oxygen at C-4′ is $H_2{}^{18}O$, while that at C-1′ is the oxygen from the sugar. At pH 9.0, therefore, the C-4′ radical appears to be sufficiently long-lived to accommodate this minor alternative pathway, which can account for increased $H_2{}^{18}O$ labeling at C-1′ and concomitant decrease in labeling at C-4′. These results are particularly intriguing given the recent report from our laboratory, which indicated that "A"-like polymers such as poly[dA-rU] gave anomalously large amounts of base release in comparison with "B" polymers upon degradation by Fe^{2+}–BLM.[23] While we postulated that Fe^{2+}–BLM could be responsible for mediating alternate chemistry at C-1′ in addition to C-4′, the mechanism in Figure 8 could also accommodate these results. Experiments are in progress to investigate this possibility and the nature of activated BLM.

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