Mechanism of Bleomycin: Evidence for a Rate-Determining 4′-Hydrogen Abstraction from Poly(dA-dU) Associated with the Formation of both Free Base and Base Propenal†

John C. Wu and John W. Kozarich*

Department of Pharmacology and Developmental Therapeutics Program, Comprehensive Cancer Center, Yale University School of Medicine, New Haven, Connecticut 06510

JoAnne Stubbe

Department of Biochemistry, College of Agriculture and Life Sciences, University of Wisconsin, Madison, Wisconsin 53706

Received May 16, 1985

ABSTRACT: When poly(dA-[4′-^{3}H]dU) was degraded by activated bleomycin under a variety of conditions, 50 ± 10% of the deoxyuridine residues were converted to uracil and uracil propenal, paralleling observations made with DNA. By manipulation of the concentration of O_2 in solution, the relative ratio of uracil propenal to uracil could be varied between 0.03 for anaerobic activation and 7.0 for activation at 3 atm of O_2. Tritium selection effects on 4′-hydrogen abstraction were also measured under these conditions and found to range from 7.2 to 12.5. These results strongly suggest that the formation of both uracil and uracil propenal is the consequence of a rate-determining 4′-carbon–hydrogen bond cleavage and of an O_2-dependent partitioning of the intermediate produced by this cleavage.

The bleomycins, a glycopeptide-derived class of antibiotics, have a well-established ability to degrade DNA. The reaction requires metal ions, such as Fe(II) or Cu(I), and O_2 and yields, as monomeric products, free nucleic acid bases and base propenals[1] (Figure 1) (Kuramochi et al., 1981; Burger et al., 1980, 1981; Sausville et al., 1976; Oppenheimer et al., 1981; Rodriguez & Hecht, 1982; Giloni et al., 1981; Ehrenfeld et al., 1985). In addition, Burger et al. (1982) have demonstrated that Fe(III) and H_2O_2 under anaerobic conditions can also activate bleomycin and yield, upon interaction with DNA, exclusively free base. Base propenal formation has been proposed to result from a radical abstraction of the hydrogen from the C-4′ position of the deoxyribose moiety by the activated bleomycin followed by formation of an unstable 4′-hydroperoxide (Giloni et al., 1981). The hydroperoxide was proposed to decompose to afford DNA strand scission and the base propenal. Subsequent important studies by Burger et al. (1982) suggested that base propenal formation was stoichiometric with DNA scission and required additional O_2. Free base release appeared to require no additional O_2 beyond that required for bleomycin activation and was stoichiometric with the formation of alkaline-labile sites.

Using poly(dA-dU), specifically tritiated at defined positions of the deoxyribose of the deoxyuridine, as a DNA model probe, we reported a bleomycin-catalyzed labilization of the 4′-hydrogen in the presence of Fe(II) and O_2 (Wu et al., 1983). These results also suggested a potentially large primary selection effect against tritium abstraction from the 4′-position. At that time we were unable to resolve whether the abstraction resulted only in the formation of base propenal, as previously proposed by Giloni et al. (1981), or led to the production of both base propenal and free base. The reason for the ambiguity rested on the fact that both calculated isotope effects were experimentally possible. In this paper we resolve this ambiguity in two ways. First, the conditions of the reaction of bleomycin and poly(dA-[4′-^{3}H]dU) result in a degradation of a sufficient percentage of deoxyuridine moieties as to allow an unambiguous determination of the isotope effect by measurement of the specific activity of the residual deoxyuridine. Second, we establish that the isotope effect for 4′-hydrogen abstraction does not vary in a series of different reaction conditions that produce a spectrum of relative yields of free uracil and uracil propenal.

Materials and Methods

dATP, dUTP, poly(dA-dU), phosphodiesterase I, and phosphodiesterase II were purchased from P-L Biochemicals. Malondialdehyde bis(dimethyl acetal) was from Aldrich. Alkaline phosphatase (calf intestine) and 2-thiobarbituric acid were obtained from Sigma. DNA polymerase I, DNA polymerase I large fragment (Klenow fragment), and exonuclease III were purchased from New England Biolabs. [5-^{3}H]dUTP was obtained from Moravek Biochemicals. Ribonucleoside-triphosphate reductase from *Lactobacillus leichmanii* was purified according to a modification of published procedures (Harris et al., 1984). Uridine phosphorylase (9.3 units/mg) and purine-nucleoside phosphorylase (40 units/mg) were the gifts of Dr. T. A. Krenitsky, Wellcome Research Laboratories (Ator et al., 1983). *S*-Adenosylhomocysteine hydrolase (SAH hydrolase, 0.65 unit/mg) was the gift of Dr. R. H. Abeles, Brandeis University. Bleomycin (Blenoxane) was the gift of Bristol Laboratories.

† Supported by NIH Grants CA-28852 and GM-34454 and American Cancer Society Grant IN-35. J.W.K. is an American Cancer Society Faculty Research Awardee (1983–1988), and J.S. is an NIH Research Career Development Awardee (AM 01222) (1983–1988).

* Address correspondence to this author at the Department of Chemistry and Biochemistry, University of Maryland, College Park, MD 20742.

[1] Trivial names and abbreviations: base propenal, the poly(dA-dU) products 3-(uracil-1′-yl)-2-propenal and 3-(adenin-9′-yl)-2-propenal; EDTA, ethylenediaminetetraacetic acid; HPLC, high-pressure liquid chromatography; AdoCbl, (5′-deoxyadenosyl)cobalamin; EHNA, *erythro*-9-(2-hydroxy-3-nonyl)adenine; SAH hydrolase, *S*-adenosylhomocysteine hydrolase.

0006-2960/85/0424-7562$01.50/0

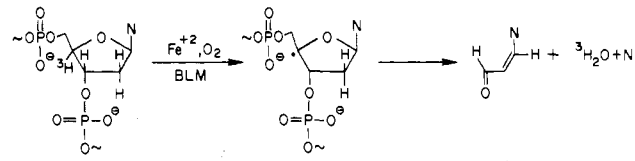

FIGURE 1: 4′-Hydrogen abstraction by bleomycin to form free base and base propenal from DNA. When the 4′-position is labeled with tritium, ^3H_2O is also produced.

Synthesis of [4′-^{3}H]dUTP. (*A*) *Preparation of [4′-^{3}H]-Adenosine.* A typical reaction mixture of 0.422 mL contained 0.4 mL of ^3H_2O (1 Ci/mL), 3 mM EHNA, 0.5 M potassium phosphate (pH 7.3), and 0.08 unit of SAH hydrolase. After 15 min, 2.1 mg of adenosine (previously lyophilized from H_2O) was added, and the mixture was incubated overnight. The adenosine slowly dissolved, resulting in a homogeneous solution by the end of the incubation period. The ^3H_2O was then removed by bulb to bulb distillation. This process was repeated until H_2O in the distillate had no detectable radioactivity. The purity of the adenosine was determined by HPLC using an ODS-1, C_{18} reverse-phase column with 12% methanol–water as the mobile phase (compound, retention time: inosine, 3 min; adenosine, 9 min). The specific activity of the recovered adenosine was 4×10^6 cpm/μmol.

(*B*) *Preparation of [4′-^{3}H]Uridine.* A typical incubation mixture of 2.0-mL final volume contained 5.0 mM [4′-^{3}H]-adenosine (4×10^6 cpm/μmol), 50 mM uracil, 0.02% NaN_3, 1 mM potassium phosphate (pH 7.4), 1.4 units of uridine phosphorylase, and 0.8 unit of purine-nucleoside phosphorylase. Uridine phosphorylase and purine-nucleoside phosphorylase were desalted by placing 2 μL (1.4 units) of the former and 2 μL (0.8 unit) of the latter in an Eppendorf tube that was centrifuged for 1 min. The supernatant was removed with a paper tissue and the pellet resuspended in 50 μL of 1 mM potassium phosphate (pH 7.4). The resulting solution was added to the above reaction mixture, which was allowed to incubate for 3 days at 37 °C. At that time the reaction had reached equilibrium as judged by HPLC analysis on ODS-1, C_{18} reverse-phase chromatography [H_2O for 9 min followed by a 0–30% CH_3OH gradient for 5 min (flow rate 1.7 mL/min); compound, retention time: uracil, 3.8 min; uridine, 4.3 min; adenosine, 10.1 min; adenine, 11.1 min]. The pH of the solution was then adjusted to 10.8 with 1 M NH_4OH and the entire mixture loaded onto a 0.5 × 5 cm Dowex-1 formate column. The column was washed with H_2O until A_{260} reached base line. This procedure removed 4.2 μmol of adenosine that was free of adenine, uracil, and uridine as judged by HPLC analysis. The uridine, uracil, and adenine were eluted from the Dowex column with 75 mL of 0.1 M ammonium formate. The effluent was concentrated in vacuo and placed on a Sephadex G-10 column (1.5 × 50 cm) in H_2O. [4′-^{3}H]Uridine was separated from uracil and adenine by this procedure, resulting in the recovery of 4.3 μmol (4×10^6 cpm/μmol), in 43% yield on the basis of adenosine.

The uridine was converted to 5′-UMP by the $POCl_3$/PO(OEt)$_3$ procedure of Yoshikawa et al. (1967). The monophosphate was converted to the triphosphate with bis(tri-*n*-butylammonium)pyrophosphate and 1,1′-carbonyldiimidazole (Hoard & Ott, 1965).

(*C*) *Conversion of [4′-^{3}H]UTP to [4′-^{3}H]dUTP.* A typical reaction mixture of 0.5 mL contained 30 mM dithiothreitol, 1 mM EDTA, 0.5 M potassium phosphate (pH 7.8), 1 M NaAc, 12 μM AdoCbl, 1.5 μmol of [4′-^{3}H]UTP (8×10^5 cpm/μmol), and 0.33 mg of ribonucleoside-triphosphate reductase from *L. leichmanii* (0.12 μmol min^{-1} mg^{-1} with UTP). The reaction was incubated for 3 h at 37 °C. To ensure 100% conversion of UTP to dUTP, an aliquot (1 μL) was removed and treated with 100 μL of 0.06 M ethanolamine (pH 10.5) containing 2 units of *Escherichia coli* alkaline phosphatase for 1.5 h at 37 °C. The mixture was analyzed for uridine and deoxyuridine on an ODS-1, C_{18} reverse-phase HPLC column with H_2O as the mobile phase (compound, retention time: uridine, 8 min; deoxyuridine, 15 min). The reaction mixture was then diluted and placed on a DEAE-Sephadex A-25 column (1.5 × 17 cm) and eluted with a 500-mL linear gradient from 0 to 0.8 M triethylammonium bicarbonate. The appropriate dUTP fractions were pooled and desalted in vacuo.

Synthesis of Poly(dA-dU) Copolymer. Poly(dA-dU) was prepared by the action of the large fragment of *E. coli* DNA polymerase I under the conditions of Setlow et al. (1972). Reactions contained 67 mM potassium phosphate (pH 7.4), 6.7 mM magnesium chloride, 1 mM 2-mercaptoethanol, ≥30 μM poly(dA-dU) as primer, 0.25 mM dATP, 0.25 mM dUTP or [4′-^{3}H]dUTP, and DNA polymerase I large fragment (10 units/mL). The reactions were maintained at 37 °C and were monitored by the decrease in absorbance at 260 nm in a 3-mm path-length cell. When the absorbance ceased to decrease (2–10 h depending on the length and concentration of primer used), EDTA was added (10 mM final concentration) and the DNA was washed with two-thirds volume of phenol equilibrated in 10 mM Tris-HCl (pH 7.5) containing 1 mM EDTA. The phenol phase was extracted once with an equal volume of buffer and the aqueous portions from the two washes were combined. When the total volume of the DNA was large (>10 mL), it was first reduced by repeated extraction with 2-butanol to remove excess H_2O without any loss of DNA copolymer. Residual organic solvents were removed by ether extraction. The final DNA solution was dialyzed against 20 volumes of 1 M NaCl in Tris-HCl–EDTA buffer (10–16 h), followed by three changes of buffer without NaCl (8–10 h each). The dialyzed poly(dA-dU) was made 0.3 M in NaAc, precipitated with ethanol, and dissolved in a small volume of 10 mM sodium phosphate (pH 7.5) to give a final copolymer concentration of ≥10 mM in nucleotides ($\epsilon_{260} = 7.5 \times 10^3$ M^{-1} cm^{-1}). The copolymer was stored at 4 °C. Final yields of 60–80% incorporation of nucleotides were achieved on the basis of UV absorbance and radioactivity measurements. The size of the copolymers in any given perparation was ≥20 000 base pairs as determined by agarose gel electrophoresis (data not shown).

Determination of the Specific Activity of [4′-^{3}H]Deoxyuridine in Poly(dA-dU). Poly(dA-[4′-^{3}H]dU) was degraded into deoxyribonucleosides in a reaction mixture that contained 2–5 mM copolymer, 66 mM Tris-HCl (pH 8.1), 5 mM $MgCl_2$, 2 mM 2-mercaptoethanol, exonuclease III (300 units), phosphodiesterase I (1.5 units), phosphodiesterase II (0.1 unit), and calf intestine alkaline phosphatase (30 units). Reaction volumes varied from 100 to 200 μL. Samples were incubated at 37 °C, and the reaction was terminated (5 h) by the addition of EDTA (10 mM final concentration). The proteins were precipitated by heating the solutions at 95 °C for 10 min and were subsequently removed by centrifugation. The deoxyribonucleosides liberated by the enzymes were initially separated from partial degradation products on a SAX anion-exchange HPLC column. Elution of the column with H_2O yielded the deoxyribonucleosides, which eluted near the void volume while nucleotides and oligonucleotides were retained. The deoxyribonucleoside fraction was concentrated, and the [4′-^{3}H]deoxyuridine was purifed on a C_{18} reverse-phase HPLC column (Rainin Microsorb Short-One) by elution with 5% methanol in H_2O. The radioactivity of the purified product was measured and its specific activity determined by a con-

comitant UV absorbance reading ($\epsilon_{260} = 10.1 \times 10^3$ M^{-1} cm^{-1}).

Bleomycin–Poly(dA-dU) Reaction Conditions. To effect variations in product partitioning, five different reaction conditions were employed in these experiments:

(*A*) *Standard Reactions under Normal Atmosphere.* A typical reaction (200 μL) contained 1.0–2.0 mM poly(dA-dU), 1.2 mM bleomycin, 1.1 mM ferrous sulfate, and 10 mM sodium phosphate (pH 7.5). The reactions were initiated by the addition of freshly prepared ferrous sulfate and incubated for 30 min at 0 °C. Aliquots (50 μL) were immediately analyzed for products by HPLC. Control reactions did not contain bleomycin.

(*B*) *Incremental Addition of Fe(II).* The concentration of bleomycin in these reactions was decreased to 0.6 mM. In lieu of initiation by a single addition of Fe(II), ferrous sulfate (5.5 mM) was added in small aliquots over a period of 30–45 min at 0 °C to give a final concentration of 1.1 mM. This method produced the most consistent yields of monomeric products.

(*C*) *O_2 or Air Bubbling under Normal Atmosphere.* Reactions conditions were identical with those described in (B) but with H_2O-saturated O_2 or air constantly bubbled into the reaction solutions at a moderate rate through a 22-gauge hypodermic needle.

(*D*) *O_2 Bubbling with Increased Pressure.* Reactions were conducted in the same manner as in (C), but the reaction vessels were maintained in a modified Amicon pressure filtration chamber that permitted constant O_2 bubbling and incremental addition of Fe(II) into the reaction solutions under elevated pressures of 3 atm or greater at 0 °C.

(*E*) *Anaerobic Reactions.* Anaerobic reaction conditions were similar to those described by Burger et al. (1981). Reactions (50 μL) contained 1.0–2.0 mM poly(dA-dU), 0.6 mM bleomycin, 0.5 mM ferric ammonium sulfate, and 10 mM sodium phosphate (pH 7.5). The reactions were sealed in a septum-stoppered flask at room temperature and were constantly purged with argon filtered through Fieser's solution (Fieser, 1924). Argon-purged H_2O_2 (40 mM) was injected into the reactions in 10-min intervals over a period of 1.5 h, until a final concentration of 8 mM peroxide was achieved. Reactions were analyzed for products 30 min after the last H_2O_2 addition. Control reactions did not contain bleomycin.

Heat Treatment of Completed Reactions. To a reaction sample (50 μL) were added 0.1 M EDTA (5 μL) and 1 M NaOH (2.5 μL). The solution was heated for 1 h at 95 °C in a 2-mL sealed polypropylene tube, cooled, and then briefly centrifuged. The sample was diluted to 1 mL with 0.1 M sodium phosphate (pH 7.5) and $CaCl_2$ (0.2 M final concentration) and filtered through a Millipore syringe. The reaction H_2O was distilled and the radioactivity in the distillate determined by scintillation counting. Evaporation of sample from the sealed tubes during the heating process was found to be negligible.

Determination of the Specific Activity of Unreacted [4′-^{3}H]Deoxyuridine from Bleomycin–Poly(dA-dU) Reactions. Poly(dA-[4′-^{3}H]dU) from a completed reaction was precipitated by the addition of 0.1 volume of 3 M sodium acetate and 3 volumes of ethanol. The suspension was cooled in a dry ice–acetone bath for 5 min and centrifuged (12000*g*, 5 min). The precipitated material was dissolved in buffer and degraded into deoxyribonucleosides, whose specific activity was determined as described above.

Calculation of the Isotope Effect. The primary tritium isotope effect, k_H/k_T, was calculated in two ways (Melander & Saunders, 1980). The first method was based on measurements of the formation of product (^3H_2O); the second, on measurement of the specific activity of the unreacted [4′-^{3}H]deoxyuridine reisolated from a completed reaction. In the first case, assuming that all of the products of the breakdown of deoxyuridine from poly(dA-dU) were in the form of uracil and uracil propenal, eq 1 is valid. T_0 is the total amount of

$$\frac{k_H}{k_T} = \frac{\ln [1 - (U + UP)S_0/T_0]}{\ln (1 - T_h/T_0)} \quad (1)$$

tritium in the reaction (cpm); S_0 is the initial specific activity of the [4′-^{3}H]deoxyuridine in the copolymer (cpm/nmol); U and UP are the amounts of uracil and uracil propenal formed in the reaction (nmol), respectively; T_h is the amount of tritium released into solvent (cpm).

In the second method, eq 2 is valid. S is the specific activity

$$\frac{k_H}{k_T} = \frac{\ln [1 - (U + UP)S_0/T_0]}{\ln (S/S_0) + \ln [1 - (U + UP)S_0/T_0]} \quad (2)$$

of the unreacted [4′-^{3}H]deoxyuridine from the bleomycin-catalyzed reaction (cpm/nmol).

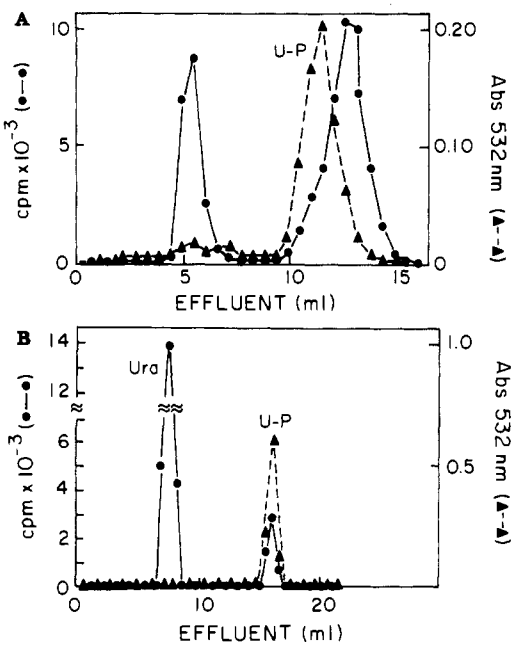

FIGURE 2: (A) Sephadex G-10 chromatography (H_2O elution) of a bleomycin–poly(dA-[5-^{3}H]dU) reaction (condition A under Materials and Methods) using tritium (●) and thiobarbituric acid (▲) analysis. (B) C_{18} reverse-phase HPLC of the low molecular weight fractions from the G-10 column in (A). A 5-μm particle size (4.6 mm × 25 cm) column was used with a gradient of 0–40% methanol in H_2O in 20 min. Flow rate was 1 mL/min.

Results and Discussion

Analysis of Products of Bleomycin–Poly(dA-dU) Reactions. Preliminary experiments using poly(dA-[5-^{3}H]dU) afforded a product profile upon reaction with bleomycin analogous to that observed for native DNA (Giloni et al., 1981). Figure 2A shows the Sephadex G-10 chromatography of a typical reaction. The column separated oligomeric products and undegraded material (4–6 mL) from the major monomeric species, uracil and uracil propenal (10–15 mL). Further fractionation by HPLC of the combined low molecular weight product peak from the G-10 column resolved uracil from uracil propenal (Figure 2B). The procedure of gel filtration chromatography followed by reverse-phase HPLC was used to obtain pure uracil propenal (λ_{max} 294 nm; $\epsilon_{294} = 3.1 \times 10^4$ M^{-1} cm^{-1}). Figure 3 shows the direct analysis of a reaction mixture by HPLC. The effluent was monitored at both 260 and 294 nm. Uracil elutes at 3 min, followed by a broad copolymer

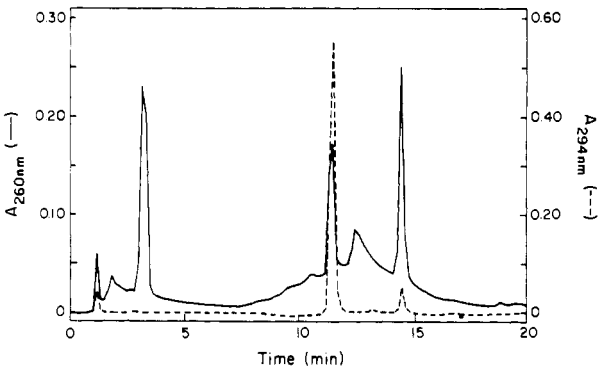

FIGURE 3: Reverse-phase HPLC of a bleomycin–poly(dA-dU) reaction (condition A) on a 3-μm particle size, Rainin Microsorb Short-One C_{18} columm (see Materials and Methods). Flow rate was 1 mL/min. Note difference in scale for A_{260} and A_{294}.

peak (9–16 min). Uracil propenal elutes in a sharp peak along with the copolymer at 12 min. This contamination does not affect the quantitation of uracil propenal because the comigrating DNA does not have measurable absorbance at 294 nm nor does it significantly react with thiobarbituric acid. Finally, adenine propenal (λ_{max} 260 nm) occurs at 14 min. Adenine elutes at 19 min and, in most cases, was negligible in amount. Normalization of the extinction coefficients and absorption maxima for the uracil- and adenine-containing products yields a relative product ratio of approximately 8:1, respectively. Bleomycin elutes from this column only after extensive washing by methanol.

Effect of Oxygen Concentration on the Partitioning between Uracil and Uracil Propenal. In light of the report of Burger et al. (1982) that bleomycin can be activated in an anaerobic system using Fe(III) and H_2O_2 to produce exclusively free base, we undertook to reproduce this reaction by using poly(dA-dU) as substrate. Furthermore, we examined the possibility that, in contrast to conditions of limited molecular oxygen supply, raising the amount of dissolved oxygen in a reaction may produce the opposite effect, that is, increased base propenal formation at the expense of free base production. Figure 4 shows the HPLC and product ratios of bleomycin–poly(dA-dU) reactions performed under varying degrees of oxygen tension, ranging from the anaerobic condition to 3 atm of O_2. The data demonstrate a marked dependence of the product ratio on the oxygen tension. In an anaerobic reaction activitated by the Fe(III)–H_2O_2 system (Figure 4A), uracil is the predominant product with an overall yield of 50 ± 10% on the basis of the initial amounts of deoxyuridine. Uracil propenal was nearly undetectable (<1%). At the other extreme, the HPLC of a reaction performed in a pressurized chamber at approximately 3 atm of O_2 is shown in Figure 4D. In this case, the overall yield of uracil propenal and uracil is also 50 ± 10%; however, the amount of uracil propenal exceeds that of uracil by nearly 10-fold. The actual magnitude of this ratio may be greater than this value since uracil propenal decomposes to yield uracil and malondialdehyde. Figure 4B,C presents the results of reactions conducted at intermediate O_2 concentrations. At an estimated $[O_2]$ of 0.45 mM, a product ratio of 2.0 was observed (Figure 4B). This value is representative of those found under usual aerobic conditions. When air bubbling is omitted, the product ratio varies from 0.3 to 1.0 (data not shown). When pure oxygen is bubbled through the reaction at 1 atm, the yield of uracil propenal was enhanced to about 5 times that of uracil (Figure 4C). In these cases, the extent of reaction was similar to the extreme conditions.

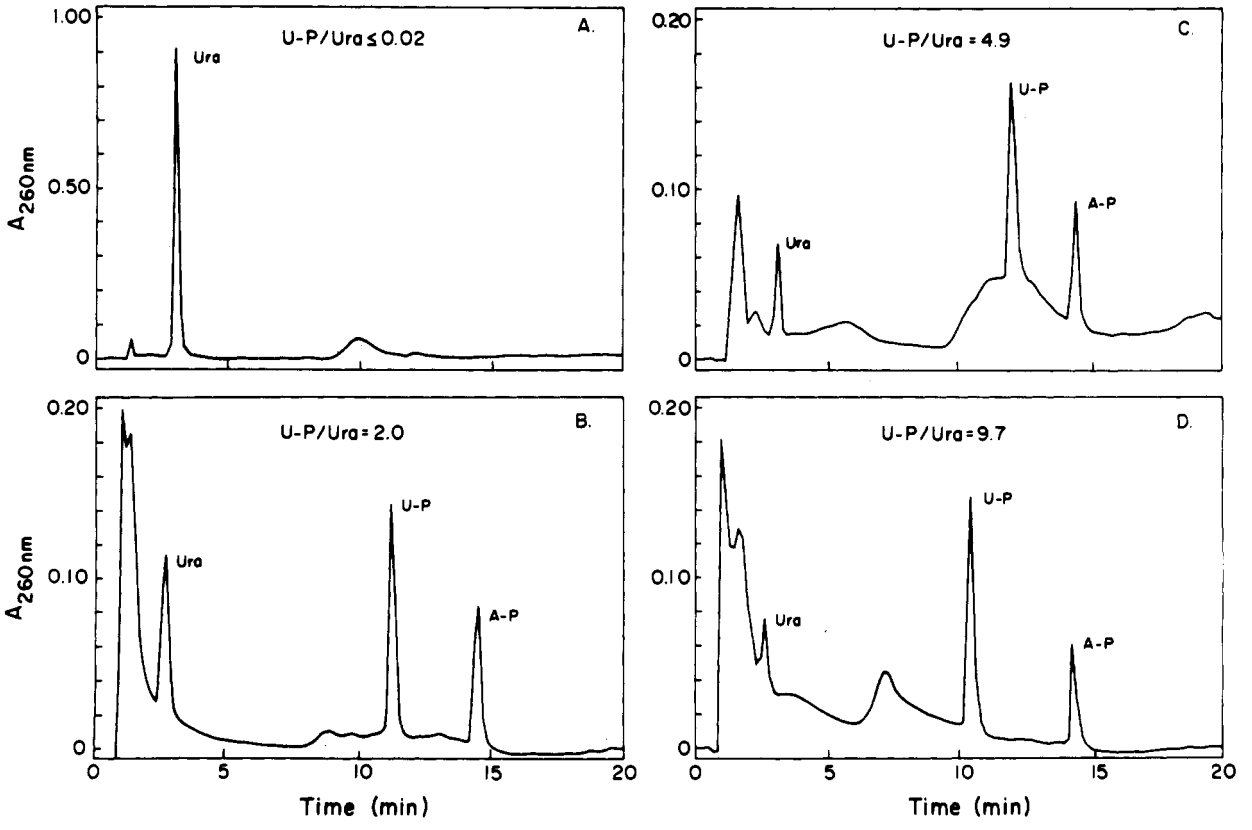

FIGURE 4: HPLC demonstrating the oxygen dependence of product partitioning between uracil and uracil propenal from poly(dA-dU) degraded by bleomycin. (A) Anaerobic reaction catalyzed by bleomycin, Fe(III), and H_2O_2 (reaction condition E under Materials and Methods). Note ordinate difference. (B) Aerobic reaction carried out with air bubbling at 1 atm (condition C). (C) O_2 bubbling under condition C. (D) O_2 bubbling with pressure increased to 3 atm (condition D). The uracil propenal to uracil ratio (U-P/Ura) for each reaction is displayed on the upper portion of the appropriate chromatogram.

Table I: Tritium Release from a Bleomycin–Poly(dA-[4′-^{3}H]dU) Reaction (+BLM) and a Control Reaction without Bleomycin (−BLM)[a]

	+BLM	−BLM
total deoxyuridine (nmol)[b]	32.2	28.6
uracil (nmol)	10.2	ND
uracil propenal (nmol)	8.9	ND
^3H_2O [cpm (nmol)][c]	530 (2.5)	15 (0.07)
^3H_2O, 95 °C, OH^- [cpm (nmol)]	599 (2.6)	23 (0.1)
k_H/k_T[d]	11.0	

[a] Condition A under Materials and Methods was used. [b] Product yields are expressed as amounts per 50-μL reaction. [c] Molar yields of tritium were calculated from an initial specific activity of 210 cpm/nmol for the [4′-^{3}H]dUrd in the copolymer starting material. [d] From eq 1.

Table II: Specific Activity (S) of Unreacted [4′-^{3}H]Deoxyuridine from Bleomycin-Treated and Control Reactions[a]

	+BLM	−BLM
total deoxyuridine (nmol)	35.2	31.4
uracil (nmol)	8.5	ND
uracil propenal (nmol)	12.4	ND
S (cpm/nmol)	513	209
S expected (cpm/nmol)[b]	478	210

[a] Values are normalized to 50-μL reaction as in Table I. [b] From eq 2 using $k_H/k_T = 11$.

The data described above support our previous proposal that free base and base propenal are formed from a common intermediate, perhaps a 4′-radical species, which can be driven to either product depending upon the availability of additional oxygen (Wu et al., 1983). Most importantly, the results afforded a variety of reaction conditions with which to examine 4′-hydrogen abstraction by using poly(dA-[4′-^{3}H]dU) as a probe for the determination of tritium selection effects.

Tritium Release into Solvent. Initial results suggested that the release of tritium from poly(dA-[4′-^{3}H]dU) was caused by direct abstraction of the 4′-hydrogen by activated bleomycin and not by a possible exchange phenomenon (Wu et al., 1983). A distinction can be made between these mechanisms of release of ^3H_2O by incubation at pH 10; the former should not be base-sensitive, while the latter should be base-catalyzed. Table I indicates that the total amount of tritium released into solvent could not be increased by vigorous treatment with heat and strong alkali. This contrasts with the results using the 3′- and 5′-tritiated copolymers, which will be discussed in the following paper (Wu et al., 1985). Thus, 4′-tritium release does not exhibit base-catalyzed exchange and is due to direct abstraction. Therefore, from eq 1 a primary isotope effect of 11 was calculated for this experiment. Control experiments indicated that neither particulate flyover during the distillation procedure nor thiobarbituric acid reactive material, which would have indicated codistillation of free malondialdehyde, was problematic.

Specific Activity of Unreacted [4′-^{3}H]Deoxyuridine. The large extent of reaction (50 ± 10%) permitted a determination of the isotope effect by measuring the increase in specific activity of the remaining substrate. The specific activity of the [4′-^{3}H]deoxyuridine isolated by the procedure described under Materials and Methods was used in subsequent calculations of the isotope effect. In Table II the specific activity of unreacted [4′-^{3}H]deoxyuridine from a bleomycin reaction is shown with the data from a control reaction performed without bleomycin. Partial degradation by bleomycin yielded deoxyuridine having nearly 2.5 times its initial specific activity. In this experiment, a fractional yield of 0.60 was measured, and according to eq 2, an expected specific activity of 478 cpm/nmol was calculated by assuming an isotope effect of 11.

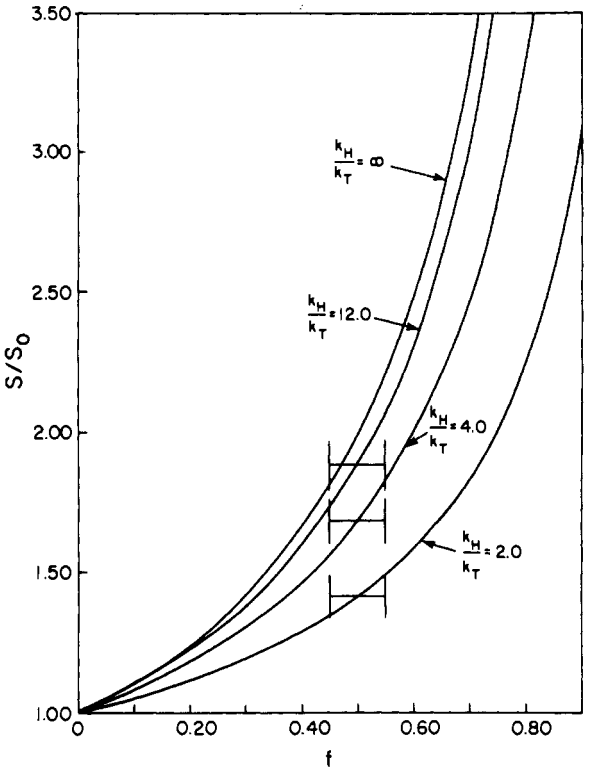

FIGURE 5: Dependence of the ratio of the final specific activity (S) to the initial specific activity (S_0) on the fractional extent of reaction (f) at different values of k_H/k_T. Horizontal bars indicate, at $f = 0.5$, a range of ±10% experimental error in product determinations. Vertical bars represent a ±5% range of values for S/S_0. Curves were derived from eq 2.

This number is 7% smaller than the experimental value of 513 cpm/nmol.

Determination of the Primary Isotope Effect for 4′-Hydrogen Abstraction. To obtain the most accurate value of k_H/k_T from our results, both eq 1 and 2 were employed for several reasons. First, because of limited materials and the nature of the bleomycin-catalyzed reaction, only completed reactions were analyzed for the formation of products, for the release of tritium, and for the specific activity of recovered starting material. This restricts the data to single points on a reaction coordinate, which maximally yields only 60% product formation. Second, the experimental error in the determination of the extent of reaction (based on spectrophotometric quantitation of uracil propenal and uracil) is approximately 10%. Third, although eq 1 is relatively insensitive to small inaccuracies in the extent of reaction, eq 2 is extremely sensitive. Figure 5 demonstrates the effect of experimental error on the calculation of k_H/k_T by eq 2. For a large selection effect (e.g., $k_H/k_T = 12$), a variability of ±10% in the fractional extent of reaction (f) and ±5% in the specific activity ratio (S/S_0) can generate values of k_H/k_T ranging from 4 to infinity. In an extreme case where the extent of reaction is underestimated by 10% (for $f = 0.5$), the value of S/S_0 would be placed beyond the theoretical limit curve ($k_H/k_T = \infty$), resulting in the calculation of a negative isotope effect by eq 2. For smaller selection effects (e.g., k_H/k_T = 2 or 4), the calculated effect will tend to stay within narrower limits. For example, for $k_H/k_T = 2$, the calculated value based on error limits described above will range from 1.5 to 3. In light of the large selection effect operative in the bleomycin reaction, our data often yielded negative isotope effects by eq 2 alone. This fact, by itself, indicates that the real isotope effect for 4′-hydrogen abstraction is greater than 6 if the limits

Table III: Determination of the Primary Isotope Effect for the Abstraction of the 4′-Hydrogen from Poly(dA-[4′-^{3}H]dU) under Various Conditions[a]

reaction condition[b]	uracil (nmol)	uracil propenal (nmol)	extent of reaction (fractional)	^3H_2O (cpm)	S (cpm/nmol)[c]	k_H/k_T (eq 1)[d]	k_H/k_T (adjusted)[e]
A (0 °C)	6.6	8.3	0.35	451	416	7.8	10.9
A (0 °C)	10.8	3.9	0.33	456	381	6.7	10.4
A (0 °C)	8.7	9.5	0.50	459	478	9.5	12.5
D (0 °C)	2.0	14.2	0.51	479	449	9.9	10.8
E (25 °C)	17.2	0.4	0.58	758	466	6.5	7.2

[a] Uracil, uracil propenal, and ^3H_2O yields are expressed as amounts per 50-μL reaction. [b] See Materials and Methods for reaction conditions A, D, and E. [c] S is the specific activity of unreacted [4′-^{3}H]deoxyuridine from the reaction. S_0 was determined individually for each experiment from a control reaction without bleomycin. The mean value of S_0 for these experiments was 195 cpm/nmol. [d] Derived from eq 1. [e] Derived from eq 1, 2, and 4 as described under Results and Discussion.

of error described in Figure 5 are enforced. The increase in the specific activity of deoxyuridine is clearly dependent upon the extent of reaction (f) as determined by the formation of both uracil and uracil propenal and not uracil propenal alone (Figure 6). The data (closed circles) correlate well with theoretical curves for large selection effects. However, the problems associated with eq 2 as discussed above result in variable k_H/k_T values.

Therefore, we chose another way to refine the calculation of the selection effect. All of the experimentally measured variables may be related to each other by setting eq 1 equal to eq 2 which yields

$$S = \frac{T_0 - T_h}{T_0/S_0 - (U + UP)} \quad (3)$$

where S is the specific activity of unreacted deoxyuridine derived from the amount of nonvolatile tritium divided by the amount of unreacted starting material (deoxyuridine). In practice, an error term (R) must be introduced to the difference between the left and right terms of eq 3 such that

$$R = \frac{T_0 - T_h}{T_0/S_0 - (U + UP)} - S \quad (4)$$

By adjusting experimental values according to R within the limits of experimental error described above, an accurate value for the selection effect can be calculated such that both eq 1 and 2 are satisfied.

Table III shows the data from five experiments performed under some of the conditions described under Materials and Methods. The k_H/k_T values in the far right column represent the best estimate on the basis of the results and eqs 1, 2, and 4. The value of 7.2 from the Fe(III)–H_2O_2 reaction is lower than that obtained from the other reactions. Unlike the other cases examined, this reaction was performed at 25 °C due to the slow rate of activation of bleomycin at 0 °C under these conditions. Theoretical calculations of the temperature dependence of large primary isotope effects suggest in general that an elevation in temperature results in a lowering in the observed effect (Vogel & Stern, 1971). In addition, kinetic differences in the activation of bleomycin by the Fe(III)–H_2O_2 system and the possibility of a different activated species might also result in differences in the observed effect. Nevertheless, the k_H/k_T of 7.2 clearly indicates a rate-determining 4′-hydrogen abstraction.

The conditions described in Table III afford a range of relative uracil propenal to uracil ratios from 0.03 [Fe(III) + H_2O_2] to 7.0 [Fe(II) + O_2 (3 atm)]. The calculated selection effects demonstrate conclusively an independence from the relative product yield of the reactions. The data yield a mean k_H/k_T of 11.1, which is within the range of known primary isotope effects for tritium. That the magnitude of this effect is unaffected by the relative yields of free base and base propenal strongly implicates the 4′-hydrogen abstraction as the rate-limiting step in the formation of both products during bleomycin-catalyzed degradation of DNA.

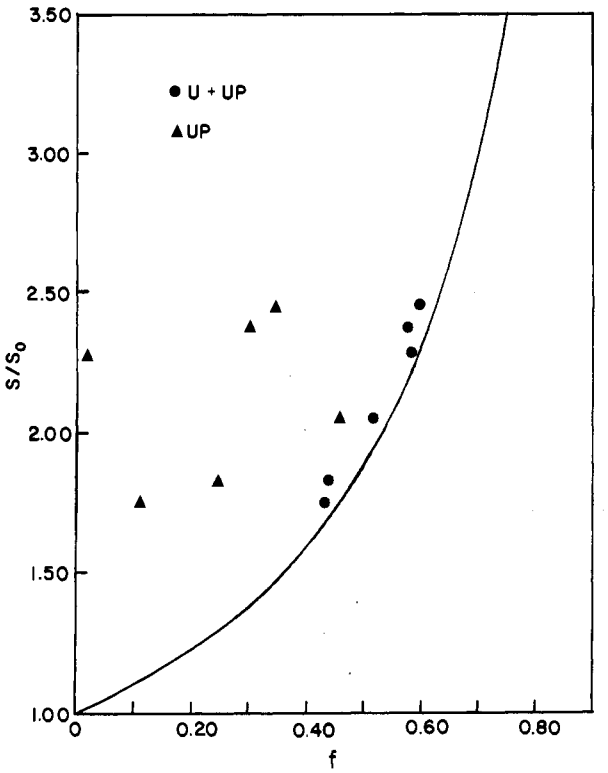

FIGURE 6: Specific activity (S/S_0) of unreacted deoxyuridine as a function of extent of reaction (f) for six representative bleomycin–poly(dA-[4′-^{3}H]dU) reactions (conditions A–E under Materials and Methods). The extent of reaction (f) is calculated for uracil propenal only (▲) and for uracil propenal and uracil (●). The theoretical curve represents a k_H/k_T of 12 generated from eq 2.

CONCLUSIONS

We have previously reported that bleomycin catalyzes the labilization of the 2′- (ribo configuration), 3′-, and 4′-hydrogens from poly(dA-dU) but not the 1′-hydrogen (Wu et al., 1983). On the basis of that evidence, we proposed that the degradation of DNA by bleomycin, Fe(II), and O_2 occurs via the initial abstraction of the 4′-hydrogen of the deoxyribose moiety to form a radical intermediate, which can yield either free base or base propenal, depending upon the mechanism of quenching of this radical (Figure 1). Recombination of the radical with an hydroxyl radical equivalent leads to the release of free base with the formation of an alkaline-labile site. The 4′-radical species upon reaction with additional O_2 yields base propenal and DNA strand scission (Giloni et al., 1981). In this paper, we have furnished evidence for the existence of an intermediate

from which both free base and base propenal are derived. The ability to drive the reaction toward a nearly exclusive yield of base propenal by the manipulation of the O_2 concentration complements the results from anaerobic reactions with Fe(III)–H_2O_2 activation (Burger et al., 1982). The overall partitioning behavior we have observed strongly suggests a common intermediate in the formation of products.

In our previous paper, we were unable to establish definitively whether the 4′-hydrogen abstraction leads to both monomeric products. The reason for this was the ambiguity in correlating the amount of 4′-tritium released with the amount of uracil propenal only or with the total amount of uracil propenal and uracil. The determination of the selection effect by measurement of the specific activity of the unreacted [4′-^{3}H]deoxyuridine is, however, unambiguous and is clearly consistent with a bleomycin-dependent, rate-determining 4′-hydrogen abstraction as the unique event leading to products. Furthermore, the relatively constant values for the selection effect under conditions yielding different product ratios confirm our hypothesis that the processes resulting in uracil or uracil propenal diverge from a common intermediate generated by the hydrogen abstraction.

We have also completed a detailed study of the bleomycin-catalyzed tritium release from poly(dA-dU) tritiated at the 3′- and 5′-positions of deoxyuridine (Wu et al., 1985). The results from those studies suggest the formation of a 4′-ketone accompanying free base release. Therefore, it seems certain that bleomycin, at least in the iron-catalyzed reaction, prefers the abstraction of the 4′-hydrogen as a first step in DNA breakage.

Registry No. Bleomycin, 11056-06-7; poly(dA-dU), 26780-70-1; uracil, 66-22-8; uracil propenal, 86798-57-4.

References

Ator, M., Stubbe, J., & Krenitsky, T. (1983) *J. Biol. Chem. 258*, 1625–1630.

Burger, R. M., Peisach, J., & Horwitz, S. B. (1980) *J. Biol. Chem. 255*, 11832–11838.

Burger, R. M., Peisach, J., & Horwitz, S. B. (1981) *J. Biol. Chem. 256*, 11636–11644.

Burger, R. M., Peisach, J., & Horwitz, S. B. (1982) *J. Biol. Chem. 257*, 8612–8614.

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

Fieser, L. F. (1924) *J. Am. Chem. Soc. 46*, 2639–2647.

Giloni, L., Takeshita, M., Johnson, F., Iden, C., & Grollman, A. P. (1981) *J. Biol. Chem. 256*, 8608–8615.

Harris, G., Ator, M., & Stubbe, J. (1984) *Biochemistry 23*, 5214–5225.

Hoard, D. E., & Ott, D. G. (1965) *J. Am. Chem. Soc. 87*, 1785–1788.

Krenitsky, T. A., Kosalka, G. W., & Tuttle, J. V. (1981) *Biochemistry 20*, 3615–3621.

Kuramochi, H., Takahashi, K., Takita, T., & Umezawa, H. (1981) *J. Antibiot. 34*, 576–582.

Melander, L., & Saunders, W. J., Jr. (1980) *Reaction Rates of Isotopic Molecules*, pp 91–127, Wiley, New York.

Oppenheimer, N. J., Chang, C., Rodriguez, L. O., & Hecht, S. M. (1981) *J. Biol. Chem. 256*, 1514–1517.

Palmer, J. L., & Abeles, R. H. (1979) *J. Biol. Chem. 254*, 1217–1226.

Rodriguez, L. O., & Hecht, S. M. (1982) *Biochem. Biophys. Res. Commun. 104*, 1470–1476.

Sausville, E. A., Peisach, J., & Horwitz, S. B. (1976) *Biochem. Biophys. Res. Commun. 73*, 814–822.

Setlow, P., Brutlag, D., & Kornberg, A. (1972) *J. Biol. Chem. 274*, 224–231.

Vogel, P. C., & Stern, M. J. (1971) *J. Chem. Phys. 54*, 779–796.

Wu, J. C., Kozarich, J. W., & Stubbe, J. A. (1983) *J. Biol. Chem. 258*, 4694–4697.

Wu, J. C., Stubbe, J., & Kozarich, J. W. (1985) *Biochemistry* (following paper in this issue).

Yoshikawa, M., Kato, T., & Takenishi, T. (1967) *Tetrahedron Lett. 50*, 5065–5068.