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DNA Degradation by Bleomycin: Evidence for 2′*R*-Proton Abstraction and for C–O Bond Cleavage Accompanying Base Propenal Formation†

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ABSTRACT: Reaction of poly(dA-[2′*S*-^{3}H]dU) with activated bleomycin yields [^{3}H]uracil propenal that completely retains the tritium label. In contrast, we have previously shown that reaction of poly(dA-[2′*R*-^{3}H]dU) with activated bleomycin affords unlabeled uracil propenal [Wu, J. C., Kozarich, J. W., & Stubbe, J. (1983) *J. Biol. Chem. 258*, 4694–4697]. We have also prepared both *cis*- and *trans*-thymine propenals by chemical synthesis and have observed that the trans isomer is the exclusive product of the bleomycin reaction. Moreover, the cis isomer was found to be stable to the conditions of bleomycin-induced DNA degradation. Taken together, these results establish that the formation of *trans*-uracil propenal occurs via an anti-elimination mechanism with the stereospecific abstraction of the 2′*R* proton. The question of phosphodiester bond cleavage during base propenal formation has also been addressed by the analysis of the fate of oxygen-18 in poly(dA-[3′-^{18}O]dT) upon reaction with activated bleomycin. The 5′-monophosphate oligonucleotide ends produced from thymine propenal formation have been converted to inorganic phosphate by the action of alkaline phosphatase, and the phosphate has been analyzed for ^{18}O content by ^{31}P NMR spectroscopy. The oxygen-18 is retained in the inorganic phosphate, establishing that the formation of thymine propenal by activated bleomycin proceeds with C–O bond cleavage at the 3′-position.

Studies from a number of laboratories indicate that the cytotoxicity of bleomycin (Umezawa et al., 1966) may be related to its ability to degrade DNA, and hence, the mechanism of nucleic acid destruction has been extensively investigated by a number of laboratories (Sausville et al., 1976; Burger et al., 1980, 1981; Kuramochi et al., 1981; Oppenheimer et al., 1981; Giloni et al., 1981; Rodriguez & Hecht, 1982; Wu et al., 1983, 1985a,b; Sugiyama et al., 1985; Murugesan et al., 1985). Their collective results have demonstrated that reaction of Fe(II), O_2, reductant, and bleomycin results in the production of a species that catalyzes the degradation of DNA to the monomeric products, base propenal[1]

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[1] Trivial names and abbreviations: base propenal, the products of bleomycin-induced degradation, 3-(uridin-1′-yl)-2-propenal and 3-(thymin-1′-yl)-2-propenal; DMF, dimethylformamide; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HPLC, high-pressure liquid chromatography; TMS, trimethylsilyl; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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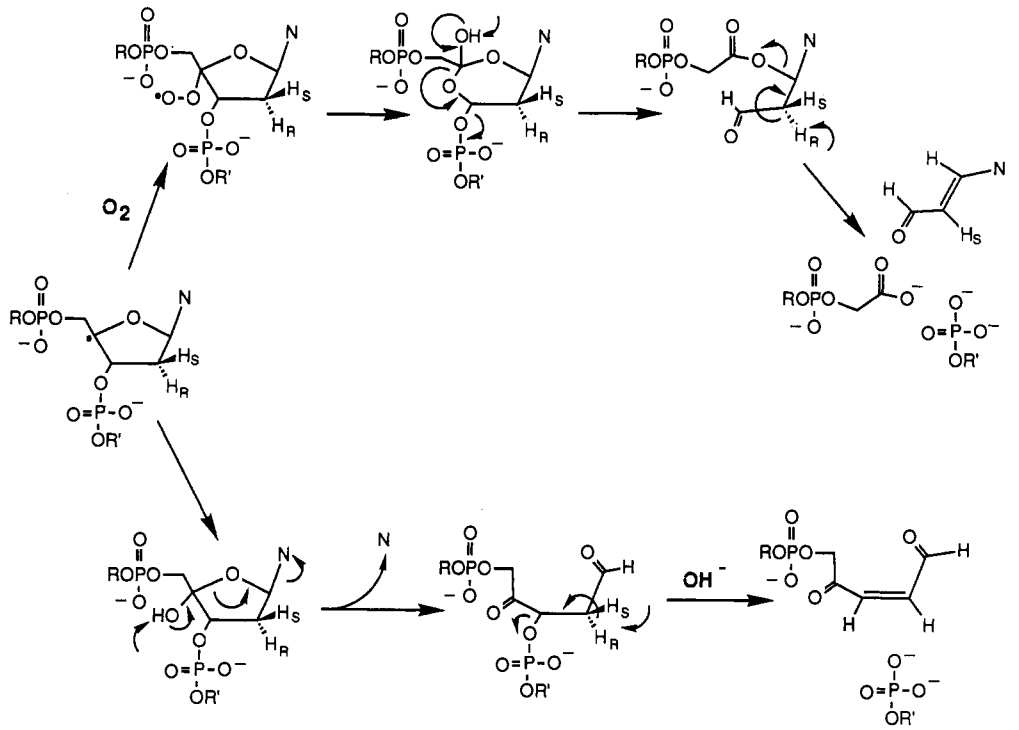

FIGURE 1: Proposed mechanism of degradation of poly(dA-dU) by activated bleomycin to form free base and base propenal.

and free nucleic acid base (Figure 1). Elegant studies by Burger et al. (1982) indicated that the production of base propenal requires O_2 in addition to that required for activated bleomycin production and is accompanied by stoichiometric DNA strand scission producing 5′-phosphate- and 3′-phosphoglycolate-modified ends. Furthermore, they demonstrated that the production of free base requires no additional O_2 and that the resultant strand scission occurs only under alkaline conditions. Additional verification of this point is provided by the observation that activated bleomycin produced by reaction with Fe(III) and H_2O_2 under anaerobic conditions yields only free base and alkaline-labile sites (Burger et al., 1982). A structure for the carbohydrate residue generated by alkaline-induced scission has been postulated (Wu et al., 1983, 1985b). Recent studies by Sugiyama et al. (1985) using oligonucleotide models provide preliminary support for this hypothesis.

These findings have suggested a mechanism for the formation of base propenal that required abstraction of the 4′-hydrogen mediated by activated bleomycin (Figure 1) (Giloni et al., 1981; Burger et al., 1982). Our studies (Wu et al., 1983, 1985a,b) utilizing specifically tritiated poly(dA-dU)'s as DNA models allowed us to verify this hypothesis and to offer a new proposal to account for the production of free nucleic acid base. We found that the rate-determining step in the production of both free base and base propenal is 4′-hydrogen abstraction, which is subject to a large tritium selection effect. Moreover, the intermediate produced by this abstraction can partition in an O_2-dependent process to afford either monomeric product. The mechanistic details of the steps following hydrogen abstraction still await elucidation.

In this paper we present further studies on the mechanism of the formation of base propenal and of the concomitant DNA strand scission. Reaction of activated bleomycin with poly(dA-[2′*R*-^{3}H]dU) or poly(dA-[2′*S*-^{3}H]dU) resulted in the abstraction of only the *pro-R* hydrogen in an anti elimination of the ribose ring oxygen to yield, exclusively, *trans*-uracil propenal. We have also shown using poly(dA-[3′-^{18}O]dT) that the DNA strand scission, generated by formation of the aldehyde group of the base propenal, occurs by C–O bond cleavage.

Materials and Methods

Tritiated $NaBH_4$ (sp act. 5.5 Ci/mmol) and $H_2{}^{18}O$ (99 atom % excess) were purchased from New England Nuclear and Stohler Isotope Chemicals, respectively. Sodium [^{18}O]acetate was prepared by the procedure of Hutchinson and Mabuni (1977). Staphylococcal nuclease was the gift of Dr. John A. Gerlt, University of Maryland. Poly(dA-[3′-^{18}O]dT), poly(dA-[2′*S*-^{3}H]dU), and poly(dA-[2′*R*-^{3}H]dU) were prepared with the Klenow fragment of DNA polymerase (Joyce & Grindley, 1983; Wu et al., 1985a). An ^{18}O-labeled poly(dA-[3′-^{18}O]dT) primer was prepared for use in the synthesis of the corresponding copolymer in order to avoid dilution of the isotopic label. Inorganic phosphate was measured by the procedure of Ames (1966). ^{1}H NMR spectra were measured on a Brüker WM 400 spectrometer. Carbon-13 and ^{31}P NMR spectra were measured at 125 and 202.4 MHz, respectively, on a Brüker WM 500 spectrometer.

[2′S-^{3}H]dUTP. The procedure of Cook and Moffatt (1967) was used to prepare 3′,5′-di-*O*-trityl-2′-ketouridine. Reduction with sodium [^{3}H]borohydride yielded a mixture that upon deblocking yielded the desired [2′-^{3}H]uridine and the labeled ara isomer in a ratio of 5:95, respectively. The uridine was purified by preparative thin-layer chromatography [EM silica gel 60 F254; solvent system: ethyl acetate–isopropyl alcohol–water (65:23:12)] and by HPLC (Rainin Microsorb C18, 5% methanol in H_2O). The position of the label has been established by ^{1}H NMR analysis of [2′-^{2}H]uridine prepared by substituting sodium borodeuteride in the above procedure. The nucleoside was converted to the corresponding 5′-triphosphate by the procedures of Yoshikawa et al. (1967) and Hoard and Ott (1965) and subsequently to [2′*S*-^{3}H]dUTP (1.56×10^5 cpm/umol) by the action of *Lactobacillus leichmannii* ribonucleotide reductase (Wu et al., 1985a).

Bleomycin–Poly(dA-[2′S-^{3}H]dU) Reaction Conditions. Poly(dA-[2′*S*-^{3}H]dU) (1.0 mM; 1.56×10^5 cpm/nmol), 0.6 mM bleomycin, 10 mM sodium phosphate (pH 7.5), and 1.1

mM ferrous ammonium sulfate were incubated at 0 °C for 30 min. Addition of the ferrous ammonium sulfate in small aliquots over this 30-min period produced the most consistent yield of monomeric products. A stream of H_2O-saturated oxygen was constantly passed through the solution from a 22-gauge hypodermic needle. The reaction was stopped by the addition of EDTA (final concentration of 10 mM), which lowered the pH to 5.5, and the mixture was divided into four 50-μL aliquots.

Fifty microliters of this sample was added to 450 μL of H_2O and was shell-frozen and subjected to bulb to bulb distillation. The amount of radioactivity recovered in the distillate was determined by scintillation counting. A second 50 μL of the sample was adjusted to pH 10 by addition of 2.5 μL of 1 M NaOH, and the sample was heated in a sealed polypropylene tube for 1 h at 95 °C. The sample was subjected to bulb to bulb distillation, and the amount of radioactivity was determined. A third 50-μL aliquot was made 0.1 M in $NaBH_4$ and incubated at 0 °C for 30 min. The pH was adjusted to 10 with 1 M NaOH and worked up as described for aliquot 2. Control experiments indicated that neither particulate flyover nor malondialdehyde was responsible for the tritium measured in the distillates. The fourth 50-μL aliquot was analyzed by HPLC using a C-18 reverse-phase HPLC column (Rainin Microsorb Short-One, 3 μm) and a 0–100% linear gradient of $H_2O \rightarrow CH_3OH$, over 20 min. Uracil had a retention time of 3 min and was quantitated by A_{260nm} ($\epsilon = 8.2 \times 10^3$ M^{-1} cm^{-1}), and uracil propenal had a retention time of 12 min and was quantitated by both A_{294nm} ($\epsilon = 3.1 \times 10^4$ M^{-1} cm^{-1}) and the thiobarbituric acid method, A_{532nm} ($\epsilon = 1.6 \times 10^5$ M^{-1} cm^{-1}) (Burger et al., 1980).

Bleomycin–Poly(dA-dU) Reaction in ^3H_2O. Poly(dA-dU) (1.3 mM), 1.1 mM bleomycin, 0.1 M sodium phosphate (pH 7.5), 1.1 mM ferrous ammonium sulfate, and ^3H_2O (5×10^6 cpm/μmol; 500-μL total volume) were incubated at 0 °C for 30 min according to the procedure described above. The reaction mixture was subjected to Sephadex G-10 chromatography (H_2O elution) as described previously (Wu et al., 1985a). The uracil propenal fraction (determined by A_{294nm} was concentrated in vacuo and purified by C-18 reverse-phase HPLC as described above. The radioactivity present in the purified uracil propenal was determined. Control experiments with uracil propenal in ^2H_2O have verified that no significant exchange of the uracil propenal hydrogens occurs during the purification (data not shown).

Synthesis of trans- and cis-Thymine Propenal. To a mixture of thymine (2.1 g, 16.7 mmol) and chlorotrimethylsilane (3.7 g, 34.0 mmol) in benzene (50 mL) was added dropwise over 1 h triethylamine (4.6 mL, 34.0 mmol) in benzene (10 mL). The reaction was maintained at room temperature for a additional 7 h. The mixture was filtered, and the filtrate was concentrated in vacuo. Purification by vacuum distillation (84 °C; 3 mmHg) afforded bis(trimethylsilyl)thymine as a clear oil (Kotick et al., 1969).

Bis(trimethylsilyl)thymine (0.13 g, 0.48 mmol) and propargylaldehyde (170 μL, 2.4 mmol) were combined neat and stirred at room temperature for 1 h (Giloni et al., 1981). The mixture was dissolved in ethyl acetate (2 mL) and purified by preparative TLC [ethyl acetate–chloroform (2:4)]. Two thiobarbituric acid reactive bands were obtained corresponding to the *cis*- and *trans*-thymine propenals [R_f 0.36 (trans) and 0.30 (cis)]. The trans isomer ^1H NMR (CD_3OD; HDO reference, 4.4 ppm) showed the following: δ 9.04 (1 H, d, J = 7.8 Hz), 7.69 (1 H, d, J = 14.6 Hz), 7.35 (1 H, s), 5.91 (1 H, q, J = 7.9 and 14.6 Hz), and 1.55 (3 H, s). NMR spectrum assignments for the cis isomer are listed below.

Production of cis-Thymine Propenal via Photolysis. The *trans*-thymine propenal in methanol (1 mM) was photolyzed at room temperature for 10–20 min with a Hanovia lamp and a Pyrex filter. This resulted in the maximum production of a 7:3 ratio of cis isomer to trans isomer, determined by isolation of the isomers by HPLC and quantitation by the thiobarbituric acid assay. In addition, thymine was also produced during this photolysis. The *cis*-propenal, isolated by HPLC, was characterized by UV and NMR spectroscopy. UV (CH_3OH:H_2O, 24:76) showed λ_{max} 275 nm. NMR (CD_3OD) gave two products a and b: (a) δ 9.34 (1 H, d, J = 6.8 Hz), 6.91 (1 H, s), 6.89 (1 H, d, J = 9.5 Hz), 5.40 (1 H, q, J = 6.8 and 9.5 Hz), and 1.47 (3 H, s); (b) δ 7.01 (1 H, s), 6.04 (1 H, d, J = 9.2 Hz), 5.08 (1 H, q, J = 6.5 and 9.2 Hz), 4.7 (1 H, d, J = 6.5 Hz), and 1.40 (3 H, s). In D_2O, the cis isomer gave an NMR spectrum for a single coupound: δ 9.33 (1 H, d, J = 7.8 Hz), 7.25 (1 H, s), 7.01 (1 H, d, J = 8.6 Hz), and 5.60 (1 H, q, J = 7.8 and 8.2 Hz). In [$^2H_6$]dimethyl sulfoxide, the cis isomer NMR spectrum was as follows: δ 9.34 (1 H, d, J = 8.1 Hz), 7.42 (1 H, s), 6.94 (1 H, d, J = 9.5 Hz), 5.34 (1 H, q, J = 7.4 and 8.9 Hz), and 1.50 (3 H, s).

Products from either photolysis or the reaction of bis(trimethylsilyl)thymine and propargylaldehyde were analyzed by HPLC with reverse-phase chromatography (flow rate 1 mL/min; 24% aqueous methanol; thymine, 4.75 min; *cis*-thymine propenal, 6.7 min; *trans*-thymine propenal, 9.0 min).

Stability of cis-Thymine Propenal under Bleomycin–Poly(dA-dU) Reaction Conditions. *cis*-Thymine propenal (0.25 mM), 1.0 mM bleomycin, 1.0 mM ferrous ammonium sulfate, and 10 mM potassium phosphate (pH 7.5; total volume 100 μL) were maintained at 0 °C in the presence and absence of 1.0 mM poly(dA-dU). At times of 0 and 30 min, a 48-μL sample was removed and immediately analyzed by HPLC as described above. The amount of base propenal was quantitated by the thiobarbituric acid assay (Burger et al., 1980).

Synthesis of [3′-^{18}O]Thymidine 5′-Triphosphate. Thymidine was converted into 5′-*O*-trityl-3′-*O*-mesylthymidine (Michelson & Todd, 1957). A mixture of the blocked thymidine (5 g, 8.88 mmol) and $CH_3C^{18}O_2Na$ (5 g, 58.08 mmol) (Hutchinson & Mabuni, 1977) in 150 mL of dry DMF was refluxed for 30 h. The reaction mixture was concentrated in vacuo and treated with 100 mL of water. Filtration and the concentration in vacuo gave a residue that was purified by chromatography on silica gel (200 g) with $CHCl_3$–MeOH (95:5) as eluant to afford 5′-*O*-trityl-3′-*O*-acetyl[3′-^{18}O]thymidine: yield 2.8 g (59.7%). This reaction proceeds with retention of configuration at the 3′-carbon via a 2,3′-cyclothymidine intermediate. This compound was treated with 100 mL of ether saturated with dry HCl gas at 0 °C for 2 h. The product was filtered, washed with ether, dissolved in 200 mL of methanol saturated with ammonia, and kept at 4 °C for 12 h. Evaporation to dryness and recrystallization from ethanol afforded [3′-^{18}O]thymidine: yield 1.1 g (85.5%); ^{13}C NMR (proton decoupled; D_2O) δ 70.42 ($C_{3'}$–^{16}O) and 70.40 ($C_{3'}$–^{18}O) (integral ratio $C_{3'}$–^{18}O:$C_{3'}$–^{16}O, 4:1); mass spectrum, m/z (M^+) 242.1 (^{16}O) and 244.1 (^{18}O) (relative intensity ^{18}O:^{16}O, 4:1).

The labeled thymidine was converted to the 5′-triphosphate according to the procedures described above.

Isolation of [3′-^{18}O]Thymidine 3′-Monophosphate from Poly(dA-[3′-^{18}O]dT). The reaction mixture contained in a final volume of 2 mL 30 mM copolymer, 50 mM Tris-HCl (pH 8.8), 10 mM $CaCl_2$, and 100 μg of homogeneous staphylococcal nuclease. The DNA was denatured by heating

at 95 °C for 10 min prior to the addition of enzyme. The reaction was incubated at 45 °C for 80 min and chromatographed on a DEAE-Sephadex A-25 column (1 × 20 cm; 160 mL linear gradient of 0–0.6 M triethylammonium bicarbonate). Fractions containing 3′-[3′-^{18}O]TMP were collected and concentrated in vacuo. The residue that was contaminated with a small amount of 3′-AMP was rechromatographed under identical conditions.

The isolated 3′-[3′-^{18}O]TMP was analyzed by ^{31}P NMR (20% $^{2}H_2O$; 2 mM EGTA): δ 3.97 (P–^{18}O) and 3.99 (P–^{16}O) (relative intensities of 73:27, respectively). For use in ^{31}P NMR, glassware, pipets, and NMR tubes were soaked in nitric acid and rinsed in distilled, deionized H_2O. Each sample was passed through a 0.5-mL Chelex 100 column (Na^+ form), and the effluent was collected directly into a 5-mm NMR tube. 3′-[3′-^{18}O]TMP (10 μmol) was incubated in 50 mM Tris-HCl (pH 8.5), 8 mM $MgCl_2$, and of 2 μL of alkaline phosphatase (Sigma Chemical Co.; *Escherichia coli*, type III; 0.36 unit/μL) in a final volume of 0.30 mL for 80 min at room temperature. The amount of inorganic phosphate formed was determined by the method of Ames (1966). The completed reaction was eluted through a 0.5-mL Chelex 100 column. The effluent was heated to 95 °C for 15 min and eluted through a second Chelex 100 column. The sample was collected in a 5-mm NMR tube and prepared for ^{31}P NMR (20% $^{2}H_2O$): δ 3.25 ($P^{16}O_4^{2-}$).

Isolation of Inorganic Phosphate from Poly(dA-[3′-^{18}O]dT) Degraded by Bleomycin. The reaction mixture contained in a final volume of 27 mL 2 mM poly(dA-[3′-^{18}O]dT), 0.6 mM bleomycin, 50 mM sodium cacodylate (pH, 7.4), and 1.1 mM ferrous sulfate added in small increments over 50 min. Reaction conditions were similar to those described above.

After 60 min, 3 mL of 3 M sodium acetate and 90 mL of EtOH were added. The solution was cooled in a dry ice–acetone bath for 5 min and centrifuged at 9000*g* for 20 min to pellet the DNA. The precipitate was dissolved in 2.5 mL of 50 mM Tris (pH 8.1) and 8 mM $MgCl_2$. Sodium acetate (0.3 mL of 3 M) was added and the ethanol precipitation procedure repeated. The pellet was washed several times with 70% EtOH, dried in vacuo, redissolved in 2 mL of Tris-HCl (pH 8.0) containing 8 mM $MgCl_2$, and incubated with 1.8 units of alkaline phosphatase for 90 min at 42 °C. EDTA (0.1 M, 100 mL) was then added to solubilize any precipitated phosphate salts. The mixture was chromatographed on a DEAE-Sephadex A-25 column (1 × 20 cm) with a 100-mL linear gradient of 0 → 0.6 M triethylammonium bicarbonate as eluant. The fractions containing inorganic phosphate were pooled and the salts removed in vacuo. The amount of P_i isolated was 6.6 μmol. The amount of thymine propenal was also determined by the method of Burger et al. (1980) and found to be 7.8 μmol. ^{31}P NMR (20% $^{2}H_2O$) showed the following: δ 3.32 ($P^{18}O^{16}O_3^{2-}$) and 3.34 ($P^{16}O_4^{2-}$) (relative intensity ^{18}O:^{16}O, 58:42).

Results and Discussion

Stereospecificity of Base Propenal Formation. Our previous studies indicated that incubation of poly(dA-[2′*R*-^{3}H]dU) with activated bleomycin resulted in the production of uracil propenal that contained no radiolabel. Furthermore, the amount (3.6 nmol of $^{3}H_2O$ and 3.1 nmol of uracil propenal) of $^{3}H_2O$ produced during this reaction was approximately equivalent to the amount of uracil propenal produced. This tritium loss from the 2′-^{3}H copolymer could have occurred by several mechanisms as indicated in Figure 2: (a) by an exchange mechanism from an intermediate step in production of uracil propenal or (b) by stereospecific loss at the *pro-R*

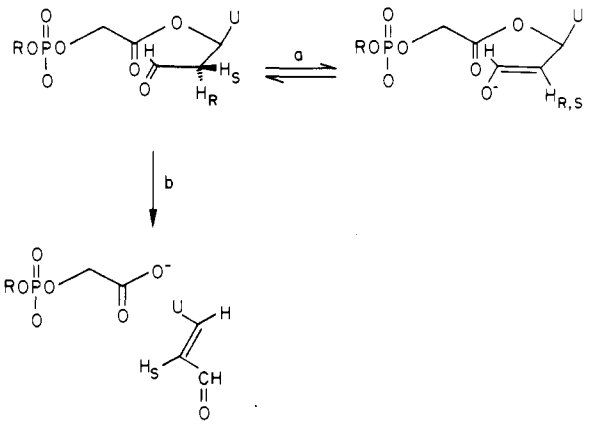

FIGURE 2: Possible mechanisms of tritium loss from 2′-^{3}H-labeled copolymer during uracil propenal formation: (a) an exchange mechanism from an intermediate step; (b) a stereospecific loss of the *pro-R* proton.

Table I: Products of Poly(dA-[2′*S*-^{3}H]dU) with Activated Bleomycin[a]

total uridine [nmol (cpm)][b]	26.8 (4166)
uracil propenal (nmol)	11.00
uracil	3.00
$^{3}H_2O$ (OH^-, 95 °C) [nmol (cpm)][c]	3.26 (508)
$^{3}H_2O$/uracil molecular ratio	1.08
$^{3}H_2O$ (OH^-, 95 °C after $NaBH_4$) [nmol (cpm)][c]	0.2 (25)
sp act. of deoxyuridine in the copolymer (cpm/nmol)	156
sp act. of uracil propenal (cpm/nmol)	153

[a] See Materials and Methods for procedure. [b] Product yields are expressed as amounts per 50-μL reaction. [c] Determined by bulb to bulb distillation.

hydrogen from the C-2′ position, leading to uracil propenal. Washout of ^{3}H from the uracil propenal can be eliminated by the lack of exchangeabiity of the propenal protons as determined by Giloni et al. (1981) using ^{1}H NMR spectroscopy and confirmed by us.

To distinguish between these mechanistic possibilities, two types of experiments were performed. In the first experiment, activated bleomycin and poly(dA-dU) were incubated in $^{3}H_2O$ (5×10^6 cpm/μmol). The base propenal produced was isolated and found to contain no detectable radioactivity. These results are inconsistent with exchange mechanism a (Figure 2a), which predicts that the recovered uracil propenal should be radiolabeled. A consequence of mechanism b (Figure 2) is that incubation of poly(dA-[2′*S*-^{3}H]dU) with activated bleomycin should result in production of uracil propenal whose specific activity is identical with that of the starting copolymer. The results of this second experiment, shown in Table I, confirm out expectations that base propenal formation occurs via stereospecific abstraction of the *pro-R* hydrogen.

Interestingly, during the degradation of the 2′*S*-^{3}H copolymer, a small amount of $^{3}H_2O$ was also produced. Since it cannot be associated with production of uracil propenal, it must be derived from production of uracil. In the pathway we have previously proposed for production of the free base (Figure 1), the 2′-protons are ultimately adjacent to the 1′-aldehyde and are, therefore, subject to exchange. To verify if $^{3}H_2O$ production was the result of an exchange process, we determined the time course of 2′-tritium release under conditions that were anticipated to accelerate proton exchange. Treatment of the terminated reaction mixture at pH 10 and 95 °C resulted in the rapid release of tritium to solvent, which was complete in 20 min. As indicated in Table I, the ratio of $^{3}H_2O$ (nmol) to uracil (nmol) produced is approximately 1.0. Furthermore, the mechanism proposed in Figure 1 in-

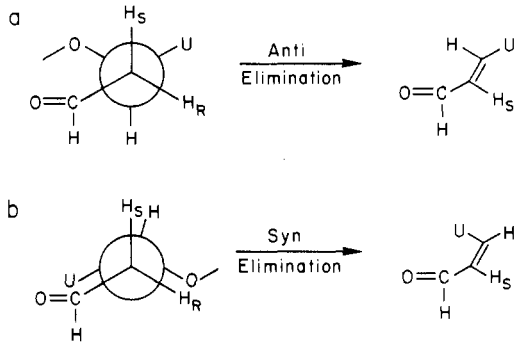

FIGURE 3: Geometry of uracil propenal formation: (a) anti elimination of *pro-R* proton and ribose oxygen to yield *trans*-uracil propenal; (b) syn elimination of *pro-R* proton and ribose oxygen to yield *cis*-uracil propenal.

dicates that this base-catalyzed exchange should be suppressed by $NaBH_4$ reduction of the aldehyde intermediate, a prediction that is confirmed experimentally (Table I). These results further support the mechanism proposed by us (Wu et al., 1983, 1985a,b) for free base release.

Geometry of Base Propenal Formation. In order to distinguish between a syn and an anti elimination of the 4′-oxygen of the ribose (Figure 3), the geometry of the uracil propenal must be established. Therefore, both *trans*- and *cis*-propenal were prepared, and their geometry was assigned by the relative magnitude of the vicinal coupling constants of the α- and β-vinylic protons with ^1H NMR spectroscopy (Giloni et al., 1981).

Two methods were used to prepare the cis isomer of thymine propenal: a modification of the coupling of (trimethylsilyl)-thymine with propargylaldehyde (Giloni et al., 1981; Johnson et al., 1984) and photoisomerization of the *trans*-propenal (Murugesan et al., 1985). Both procedures gave mixtures of products. The cis isomer could be separated from *trans*-thymine propenal and thymine by HPLC (Figure 4a). Interestingly, the cis isomer, which elutes before the trans isomer, appears as a broad peak. The ^1H NMR spectrum of the cis isomer in CD_3OD (see Materials and Methods) consists of two products in a 1:4 ratio. The minor component clearly contains a free aldehyde (δ 9.34) while the major component exhibits a resonance consistent with a hemiacetal structure (δ 4.7). The UV–vis spectrum of this mixture yields a λ_{max} of 275 nm with an ϵ_{275} of 9000 M^{-1} cm^{-1} on the basis of the thiobarbituric acid assay. ^1H NMR spectra of the cis isomer were also recorded in [$^2H_6$]dimethyl sulfoxide and in D_2O. In striking contrast to the CD_3OD spectrum, these spectra revealed a single compound consistent with the cis isomer. These observations, in addition to the absence of hemiacetal formation in the *trans*-propenal in CD_3OD, have led us to propose that the *cis*-thymine propenal (in CH_3OH) exists in equilibrium with an intramolecular hemiacetal generated by addition of the C-2 keto group of the thymine base to the aldehyde. This structure appears be present in CH_3OH and not in H_2O, an observation for which we have no immediate explanation. This equilibrium might account for the broad peak observed by HPLC chromatography (Figure 4a). The verification of this speculation does not affect the integrity of the assignment of the *cis*-propenal. Murugesan et al. (1985) reported, using a similar photolysis experiment in CH_3OH, a 95% conversion of *trans*-thymine propenal to *cis*-thymine propenal after 24 h of irradiation. In our hands, increased irradiation times >20 min resulted in destruction of both *cis*- and *trans*-thymine propenals. In addition, their HPLC results also yield a broad peak for the "cis" isomer and a λ_{max} (CH_3OH) of 298 nm. The reasons for these differences are not clear.

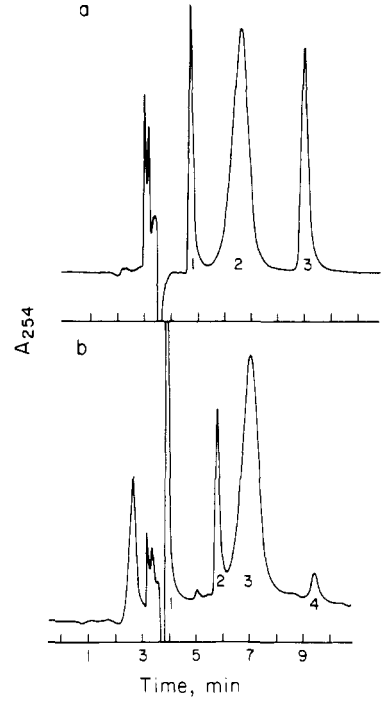

FIGURE 4: HPLC analyses of *cis*- and *trans*-thymine propenals. (a) Standards: thymine (1), *cis*-thymine propenal (2), and *trans*-thymine propenal (3). Reversed-phase C-18 HPLC chromatography: flow rate 1 mL/min; 24% CH_3OH–76% H_2O. (b) Incubation of *cis*-thymine propenal with bleomycin, Fe(II), and poly(dA-dU) (see Materials and Methods). After 30 min at 0 °C, an aliquot was injected onto a C-18 reversed-phase HPLC column at a flow rate of 1 mL/min (24% CH_3OH–76% H_2O): uracil (1), uracil propenal (2), *cis*-thymine propenal (3), and *trans*-thymine propenal (4). The ratio of *cis*- to *trans*-thymine propenal, quantitated by the thiobarbituric acid assay, was identical with that determined for time zero.

Stability of cis-Thymine Propenal. Giloni et al. (1981) established that *trans*-thymine propenal was produced during bleomycin-catalyzed degradation of DNA. However, since the *cis*-thymine propenal was not reported, it was not clear whether the conditions required for the bleomycin-catalyzed degradation of DNA could effect the rapid isomerization of the cis isomer to the more stable trans isomer. Therefore, *cis*-thymine propenal, synthesized by two independent procedures described above, was incubated with bleomycin, Fe(II), and poly(dA-dU) at 0 °C. Under these conditions, no isomerization was observed (Figure 4b). This result, in conjuction with our studies using poly(dA-[2′*R*-^{3}H]dU) and poly(dA-[2′*S*-^{3}H]dU), allows us to conclude that bleomycin-mediated production of base propenal occurs by an anti-elimination mechanism (Figure 3a). Similar results were recently reported by Murugesan et al. (1985), on the basis of their isolation and characterization of the *cis*-propenal as well as data that we previously reported (Wu et al., 1983).

Mechanism of Bleomycin-Induced Phosphodiester Bond Cleavage. At least two mechanisms have been proposed for DNA strand scission that occurs concomitant with base propenal production (Figure 5) (Hecht, 1979; Grollman et al., 1985). A distinction may be made between these possibilities by use of specifically ^{18}O-labeled poly(dA-[3′-^{18}O]dT). Incubation of this copolymer with activated bleomycin will generate a 5′-monophosphate and a 3′-phosphoglycolate for each base propenal produced. The 5′-monophosphates of the damaged copolymer may be selectively converted to inorganic phosphate (P_i) by the action of alkaline phosphatase. The occurrence or absence of ^{18}O in this P_i will permit a distinction between C–O and P–O bond cleavage to generate the 5′-phosphate (Figure 5).

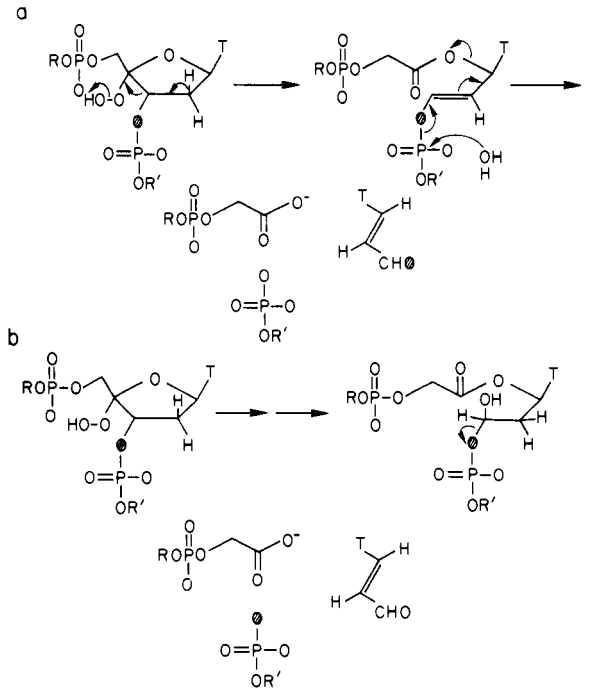

FIGURE 5: Proposed mechanisms of phosphate ester cleavage in poly(dA-[3′-^{18}O]dT) by activated bleomycin: (a) P–O bond cleavage to yield thymine [^{18}O]propenal; (b) C–O bond cleavage to yield 5′-[^{18}O]phosphate ends.

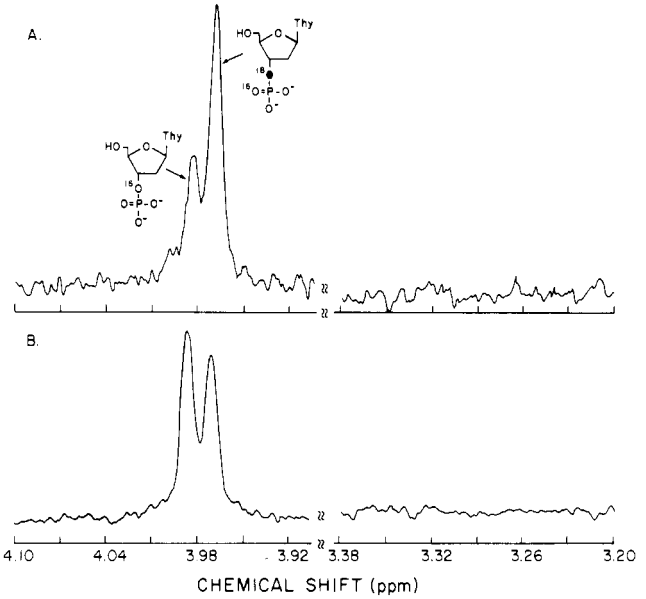

FIGURE 6: ^{31}P NMR spectra of 3′-[3′-^{18}O]TMP: (A) NMR spectrum of 3′-[3′-^{18}O]TMP generated by staphylococcal nuclease digestion of poly(dA-[3′-^{18}O]dT) (9.7 mM 3′-TMP, see Materials and Methods for sample preparation procedures); (B) NMR spectrum after the addition of an equimolar concentrtion of 3′-[^{16}O]TMP. Chemical shift values are relative to 85% H_3PO_4.

A mechanism involving P–O bond cleavage (e.g., Figure 5a) predicts that the ^{18}O present in the labeled copolymer would be found initially in the base propenal and, due to exchange of the aldehydic oxygen, ultimately in H_2O. A mechanism involving C–O bond cleavage (e.g., Figure 5b) predicts that ^{18}O would be found in the 5′-monophosphate fragment. Alkaline phosphatase treatment of this material should result in the production of ^{18}O-labeled P_i. We have used ^{31}P NMR to distinguish between C–O and P–O mechanisms of phosphodiester bond cleavage.

In order to determine the amount of ^{18}O incorporated into the poly(dA-[3′-^{18}O]dT), the copolymer was degraded with staphylococcal nuclease and the 3′-[3′-^{18}O]TMP isolated by anion-exchange chromatography. The ^{31}P NMR spectrum of this material is shown in Figure 6 and by integration contains a 26:74 mixture of ^{16}O to ^{18}O at the 3′-position. Incubation of 3′-[3′-^{18}O]TMP with *E. coli* in production of [^{16}O]P_i (data not shown), confirming that the ^{18}O is bonded directly to the 3′-carbon of the nucleotide.

Table II: Products from Poly(dA-[3′-^{18}O]dT) Degraded by Activated Bleomycin[a]

thymine (μmol)	1.56
thymine propenal (μmol)	7.80
adenine propenal (μmol)	0.32
endogenous phosphate (μmol)[b]	0.4
reaction phosphate (μmol)[c]	6.7
^{18}O:^{16}O obsd isotope ratio[d]	58:42
^{18}O:^{16}O expected isotope ratio[e]	74:26

[a] Monomeric products were determined by HPLC and expressed as total yield per 30 mL. [b] Inorganic phosphate released by alkaline phosphatase before reaction with activated bleomycin. [c] Inorganic phosphate release by alkaline phosphatase after reaction with activated bleomycin. [d] Determined from ^{31}P NMR integration. [e] Based on the expected [^{18}O]phosphate (amount of thymine propenal × 0.80) and the expected [^{16}O]phosphate (amount of thymine propenal × 0.20 plus amount of adenine propenal plus amount of endogenous phosphate).

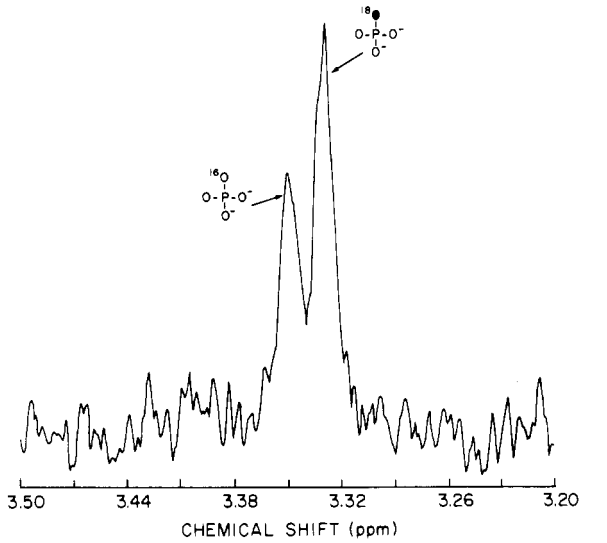

FIGURE 7: ^{31}P NMR spectra of inorganic phosphate (8.2 mM) isolated from poly(dA-[3′-^{18}O]dT) treated with activated bleomycin (see Materials and Methods for procedure). Assignment of the resonances was verified by NMR analysis of the sample after separate additions of authentic monosodium [^{18}O]phosphate and sodium [^{16}O]phosphate (data not shown).

Previous work by Burger et al. (1982) established that base propenal production is accompanied by stoichiometric DNA strand scission and the production of 5′-phosphate- and 3′-phosphoglycolate-modified ends. Therefore, incubation with alkaline phosphate should liberate P_i from the 5′-modified ends in amounts that are stoichiometric with the amounts of base propenal produced (Figure 5). Incubation of 23 μmol of poly(dA-[3′-^{18}O]dT) with bleomycin under saturating O_2 conditions and subsequent treatment with alkaline phosphatase resulted in the production of 7.8 μmol of thymine propenal and 6.7 μmol of P_i, thus establishing the validity of this approach. The high O_2 concentrations were successfully used to effect the partitioning toward base propenal and away from free base (Wu et al., 1985a) (Table II). Previous studies of Burger et al. (1982) have established that thymine production is accompanied by strand scission only in the presence of alkali. Thus, the P_i isolated in this experiment is associated only with base propenal production.

A ^{31}P NMR spectrum of the isolated P_i is shown in Figure 7 and indicates a ^{16}O:^{18}O ratio of 42:58. The result clearly establishes a C–O bond fragmentation accompanying base propenal and 5′-monophosphate formation. The washout of ^{18}O from P_i relative to the ^{18}O content in the copolymer is a function of the known exchange of phosphate oxygens with H_2O catalyzed by alkaline phosphatase (Schwartz & Lipmann, 1961). In a control experiment (data not shown) we have determined by ^{31}P NMR that the concentration of alkaline phosphatase used in this experiment is capable of catalyzing the statistical exchange of phosphate oxygens at a rate sufficient, within experimental error, to completely account for the observed washout of ^{18}O. This suggests that any alternative P–O cleavage pathway leading to base propenal is, at best, a minor contributor of this product.

Summary. These experiments have focused on the specific events in base propenal production. They are consistent with aldehyde formation in the propenal via C–O bond cleavage of the phosphodiester bond, stereospecific removal of the *pro-R* hydrogen at the 2′-position, and anti elimination of the former ribose ring oxygen to yield exclusively *trans*-base propenal. The stereospecificity of the proton abstraction is remarkable and may suggest that this process is mediated by a specifically positioned base that is part of the bleomycin–DNA complex.

Registry No. HC≡CCHO, 624-67-9; bleomycin, 11056-06-7; *trans*-thymine propenal, 79251-82-4; *cis*-thymine propenal, 97920-40-6; *trans*-uracil propenal, 103732-07-6; thymine, 65-71-4; bis(trimethylsilyl)thymine, 7288-28-0.

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