Redox Potential of Iron–Bleomycin†

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ABSTRACT: The oxidation–reduction potential of the iron complex of bleomycin, an antitumor agent that cleaves deoxyribonucleic acid (DNA), was measured at 25 °C, pH 7.0, by using both a microcoulometric and an optical absorption method. A value of 129 ± 12 mV was obtained vs. the standard hydrogen electrode for a single-electron process. Thus, ferrous bleomycin, the active form of the antitumor drug, can be regenerated under physiological conditions from ferric bleomycin, a product of the DNA cleavage reaction.

The bleomycins, a family of glycopeptide antibiotics with significant antineoplastic activity, have been isolated from *Streptomyces verticillus* as their copper chelates by Umezawa and his associates (Umezawa, 1975), who have shown that this group of drugs have similar structures and differ only in their terminal groups. The bleomycins are capable of forming 1:1 complexes with Fe(II), Zn(II), Cu(II), and other transition metal ions (Sausville et al., 1978; Dabrowiak et al., 1978). The elucidation of their structures has been the object of several investigations using a variety of physical probes (Dabrowiak et al., 1978; Takita et al., 1978; Iitaka et al., 1978; Antholine & Petering, 1979; Oppenheimer et al., 1979; Burger et al., 1981). Of the numerous metal complexes that bleomycin is capable of forming, only the Fe(II) complex is active in the in vitro cleavage of deoxyribonucleic acid (DNA)[1] (Sausville et al., 1976; Burger et al., 1979a), a reaction that also requires molecular oxygen (Ishida & Takahashi, 1975; Onishi et al., 1975; Sausville et al., 1976). An oxygenated intermediate of ferrous bleomycin has been investigated by stopped-flow spectrophotometry (Burger et al., 1979b), and it has been shown to give rise to ferric bleomycin. The latter cannot cleave DNA, but the ferrous complex can be regenerated in vitro in the presence of reducing agents such as mercaptoethanol or dithiothreitol and can subsequently carry out further DNA cleavage reactions (Sausville et al., 1978). In order to understand the redox changes that the metal–drug complex undergoes and their relationship to DNA cleavage activity, we have measured the redox potential of iron–bleomycin.

Materials and Methods

Bleomycin sulfate (Blenoxane) was a gift of Bristol Laboratories and was used without further purification. This drug was a metal-free, lyophilized preparation containing approximately 60% bleomycin A_2, 30% bleomycin B_2, and 10% various other bleomycins. Solutions were prepared the day of the experiment in metal-free, deionized, distilled water. A molecular weight of 1550 was assumed for the drug and $\epsilon_{292} = 1.45 \times 10^4\ M^{-1}\ cm^{-1}$.

Hepes[1] was obtained from Sigma Chemical Co. and prepared as a 0.05 M solution adjusted to pH 7.0 with NaOH. Phenazine methosulfate and 2,6-dichlorophenolindophenol were purchased from Aldrich Chemical Co. and Sigma, respectively, and used as mediators for the electron-transfer reactions. Ascorbic acid (Naharai Chemicals) and cysteine (Sigma), used as reductants, were prepared in Hepes buffer. Reagent grade $Fe^{II}(NH_4)_2(SO_4)_2 \cdot 6H_2O$ from J. T. Baker Chemical Co. was dissolved in distilled deionized water. All solutions were used immediately after preparation. Solutions and electrochemical cells requiring anaerobic conditions were purged with stirring and gentle agitation under a flow of ultra high purity grade argon or N_2 (Linde).

Optical Studies. Optical absorption spectra were obtained on either a Cary Model 14R or a Cary 17D recording spectrophotometer with a 1 cm pathlength cuvette or a 1 cm pathlength modified Thunberg cuvette equipped with a septum-stoppered sidearm, capable of being purged of O_2.

Sample Preparation. Fe(II)–BLM (8.6×10^{-4} M final concentration) was prepared from solutions of the drug in 0.05 M Hepes buffer, pH 7.0, or deionized distilled water. These were transferred to the body proper of the modified Thunberg cuvette. Anaerobiosis was achieved by purging with N_2. Slightly less than 1 stoichiometric equiv (90–95%) of Fe(II) was added anaerobically from the sidearm. In the case of unbuffered solutions, the pH was adjusted anaerobically to ~8 with NaOH so as to ensure that Fe(II) was completely bound to the drug (Burger et al., 1979a).[2] The extent of metal binding was deduced from the absorption at 476 nm ($\epsilon = 3.8 \times 10^2\ M^{-1}\ cm^{-1}$) (Sausville et al., 1978). Ferric bleomycin was formed from the ferrous drug complex by air oxidation. The extent of reaction was determined from the chromophore at 385 nm (Burger et al., 1979b). After oxidation, the pH was readjusted to 7.0, and the solution was again made anaerobic by N_2 purging.

Coulometric Potentiostatic Measurements. The coulometric–potentiostatic measurements were performed with a Princeton Applied Research Model 173 potentiostat–galvanostat equipped with a Model 179 digital coulometer. The instrument was operated in the current–potential mode and the current was monitored either with the coulometer or a Houston Omniscribe strip chart recorder set to follow current vs. time.

The potentiostatic cell consisted of a Plexiglas body with a tight-fitting top. A standard calomel electrode served as reference with an isolated platinum wire and a platinum grid

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[1] Abbreviations used: DNA, deoxyribonucleic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; PMS, phenazine methosulfate; BLM, bleomycin; DCPIP, 2,6-dichlorophenolindophenol; SCE, standard calomel electrode; SHE, standard hydrogen electrode.

[2] A determination was made of the volume of concentrated NaOH that was required to raise the pH of an aerobic solution of metal–drug complex.

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serving as counter and working electrodes, respectively. The cell, which contained a working volume of 4 mL of solution, was continuously purged with N_2 and stirred with a small, constant-speed mechanical stirrer. Equilibrium was achieved in ~1 h after the anaerobiosis procedure was initiated, as judged from a stable residual current.

Studies were performed in 0.05 M Hepes, pH 7.0, containing 50 μM mediator. Iron bleomycin samples were injected in 5–80-μL aliquots with a Hamilton gastight syringe. Current–time curves showed a rapid rise with an exponential decay over a 2–15-min time course. Residual currents were in the range 0.5–5 nA depending on the potential.

The current–time integrals were determined either by reading directly from the digital coulometer or by measuring the area under the recorded curve with a planimeter. The second method was preferred since it gave a record of the current–time curve which could be used to determine the rate of current decay and also the time when equilibrium was achieved. Furthermore, the shape of the curve was indicative of the condition of the electrode system. Since the residual current level can be affected by depositions on the platinum electrode, the use of the digital coulometer, even with a compensator, was of limited value.

Optical Potentiometric Measurements. The optical cells used for the potentiometric study consisted of a modified Thunberg cuvette equipped with ground glass 14/20 joints designed to accommodate an adaptor for both N_2 purging and sample injection and a standard calomel electrode. A platinum wire mesh electrode was attached through the side wall with cobalt glass. During the experiment, the cuvette was constantly purged with N_2 and stirred with a Teflon magnetic stirring bar and a solid-state motor. The platinum and reference electrodes were connected respectively to the glass and mV connectors of a Radiometer Model 52 digital pH meter. The electrodes, tubing, and stirrer were adjusted to remain outside of the optical path but in full contact with the solutions under study.

An anaerobic Fe(III)–BLM sample was reduced stepwise at pH 7.0 by injection of fractional electron equivalents of ascorbate or cysteine. The potential and visible spectrum were recorded after each addition when equilbrium was achieved, as judged by constancy of both the potential and the optical spectrum. No mediators were employed in the optical study.

Analysis of Data. The data obtained from the potentiometric titrations were analyzed by computer-iterated least-squares fits to the Nernst equation. The standard oxidation–reduction potential at pH 7.0 (A), number of electrons (n), and theoretical limit of total iron–bleomycin (B) were parameters of the algorithm

$$X = A + \frac{25.6}{n} \ln \frac{Y}{B - Y}$$

X equals the measured potential (millivolts), and Y equals the concentration of Fe(III)–BLM. The data were also analyzed with a fixed n value equal to 1 or 2, in order to determine deviations from a predicted value and to compare the standard redox potentials obtained for both methods of analysis.

Results

Reduction of Ferric Bleomycin. The potentiostatic reduction of ferric bleomycin is an extremely slow process, requiring at least 15 min to achieve equilibrium after the introduction of electrons. However, when PMS is used as a mediator ($E^{\circ\prime}$ = 80 mV) (Dickens & McIlwain, 1938), the reduction is fast. This can be readily demonstrated from a rise in current flows, followed by an exponential decay to a preexisting residual level

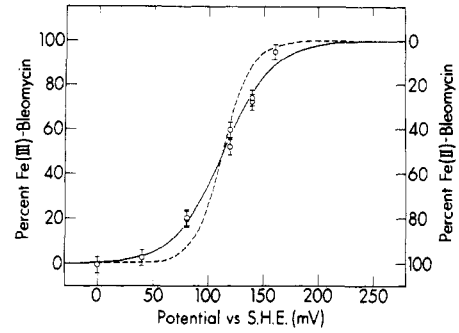

FIGURE 1: Oxidation–reduction potential of Fe(III)–BLM as determined from a potentiostatic titration. Reduction of anaerobic Fe(III)–BLM (0.86 mM) in a 50 mM Hepes buffer solution containing 0.05 mM PMS in the potentiostatic cell was monitored as described under Materials and Methods. The data are shown plotted as the percentage of Fe(III)- or Fe(II)–BLM vs. the potential as compared to the standard hydrogen electrode (SHE). The solid line is a computer-generated least-squares fit of the data to the Nernst equation, yielding an $E^{\circ\prime}$ value of 114 mV and an n value of 1.15. The least-squares fit with n = 1 (not shown) followed very closely to the solid line. A least-squares fit of the data with an enforced n = 2 (dashed line) is also shown. The mean weight error for the n = 2 fit is more than double that for n = 1. Error bars indicate the standard deviation of the data points from the function.

Table I: Redox Potential Measurements of Iron-Bleomycin[a]

method	reductant	$E^{\circ\prime}$ (mV)	n
potentiostat	electrons	131	0.97
potentiostat	electrons	114	1.15
optical	ascorbate	112	1.29
optical	ascorbate	140	0.94
optical	ascorbate	139	0.96
optical	ascorbate	145	0.96
optical	ascorbate	142	0.94
optical	cysteine	123	0.97
optical	cysteine	119	1.22
	av:	129 ± 12	1.04 ± 0.13

[a] Data obtained either by the potentiostatic method, in the presence of phenazine methosulfate, or optically, with ascorbate or cysteine as reductant, were fit by a least-squares fitting procedure to the Nernst equation. The computed redox potentials and n values are tabulated for nine individual experiments.

when the potentiostat is set to a sufficiently low potential, approximately −200 mV. In the absence of iron, bleomycin does not produce a significant current flow at this potential, either in the presence or absence of PMS.

With other mediators, such as DCPIP ($E^{\circ\prime}$ = 217 mV) (Gibbs et al., 1925), the reduction of Fe(III)–BLM is as slow as in the absence of PMS, requiring more than 15 min. However, it can be monitored by following the exponential current decay curve. Both DCPIP and PMS are readily reduced electrochemically in the absence of Fe(III)–BLM as determined from current flow and change of color.

The degree of reduction of an anaerobic solution of Fe(III)–BLM (8.6×10^{-4} M) in Hepes was measured by using the coulometric potentiostatic cell in the presence of 5.0×10^{-5} M PMS, throughout the range −80 to +300 mV, vs. the SHE. The current–time integrals were plotted against the potential after normalization to full reduction (Figure 1). The data fit to a Nernst equation yielded a value of $E^{\circ\prime}$ = 114 mV and a computed n value of 1.15. In a second experiment $E^{\circ\prime}$ was 131 mV and the n value was equal to 0.97 (Table I). These data demonstrate that the electrochemical reduction of Fe(III)–BLM is a single-electron process.

The reductive titration of Fe(III)–BLM was studied optically and monitored throughout the range −400 to +140 mV

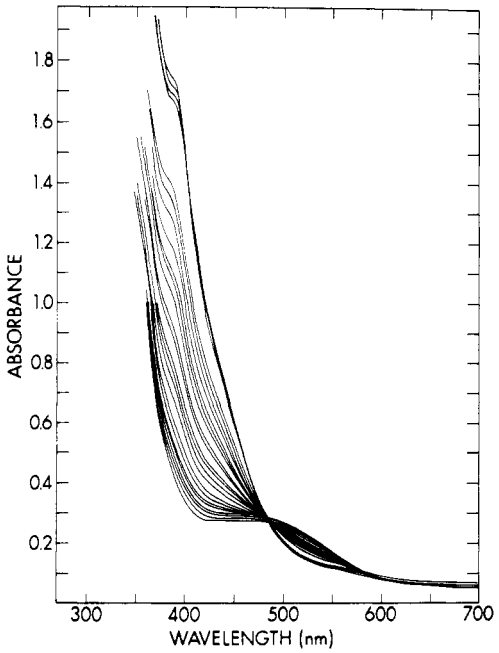

FIGURE 2: Optical redox titration of Fe(III)–BLM. In this experiment aliquots of ~20 μL of 12 mM anaerobic ascorbate solution were added successively to an anaerobic solution of 0.6 mM Fe(III)–BLM in 50 mM Hepes buffer, pH 7.0. The isosbestic near 485 nm indicates redox interconversions between two chemical species. This suggests that no long-lived spectral intermediates are produced in the reaction Fe(III)–BLM + $1e^- \rightleftarrows$ Fe(II)–BLM. Aerobic oxidation of Fe(II)–BLM (data not given) shows the same spectral changes as the reduction, and with clean isosbestics.

in Hepes by utilizing either ascorbate or cysteine to generate reducing equivalents. The reaction was followed by the loss of the chromophore attributable to Fe(III)–BLM and the concomitant generation of the chromophore of Fe(II)–BLM (Sausville et al., 1978) (Figure 2). Since the overall reaction was shown to be a one-electron process (Table I), the optical changes occurring within the potential range employed in this study cannot be due to any redox-sensitive moiety other than that giving rise to the chromophore. This warrants the interpretation that the iron is the species that is chemically reduced. This inference is further substantiated by the loss of the EPR signal of Fe(III)–BLM (Burger et al., 1979a) after reaction with reductant.

A plot of the percent of total iron present as Fe(III)–BLM against the potential was fit by a least-squares procedure to the Nernst equation (Figure 3). The results of individual experiments are given in Table I. The redox potential obtained by the optical procedure is in good agreement with the value obtained potentiostatically in the presence of PMS.

These results confirm that the redox potential that we have determined is that of a Fe(III)–BLM/Fe(II)–BLM electrochemical couple. However, the potential might be that of the Fe(III)–Hepes/Fe(II)–BLM couple and/or the Fe(III)–BLM/Fe(II)–Hepes couple, where Hepes effectively competes with bleomycin for Fe(III). To investigate this possibility, we studied the effect of Hepes buffer on the optical spectrum of Fe(II)– and Fe(III)–BLM. The optical spectrum of neither iron–drug complex was changed by the addition of 0.05 M Hepes buffer at pH 7.0. Furthermore, the EPR spectrum of Fe(III)–BLM (Burger et al., 1979a) at neutral pH is unaffected by Hepes either added before air oxidation of the ferrous complex or directly to the ferric complex (data not shown).

These results with Hepes are to be compared with analogous experiments done with phosphate buffer. Whereas 0.05 M phosphate, pH 7.0, does not affect the optical spectrum of

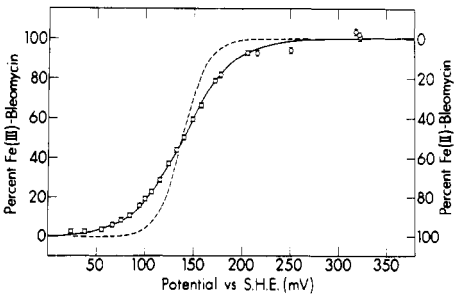

FIGURE 3: Oxidation–reduction potential of Fe(III)–BLM determined from a spectrophotometric titration. Ascorbate reduction of an anaerobic solution of Fe(III)–BLM (0.6 mM) in 50 mM Hepes buffer, pH 7.0, was followed both spectrophotometrically (see Figure 2) and potentiometrically as described under Materials and Methods. Data were plotted as the percentage of Fe(III)– or Fe(II)–BLM, determined optically, vs. the potential as compared to the standard hydrogen electrode (SHE). The curve is a computer-generated least-squares fit of the data to the Nernst equation, yielding $E^{\circ\prime}$ = 139 mV with an n value of 0.96. A least-squares fit with a fixed value of n = 1 yielded a curve very similar to the solid line. A least-squares fit with a fixed value of n = 2 (dashed line) is also shown. The mean weight error for the n = 2 fit is more than double that for n = 1.

Fe(II)–BLM, it does alter the optical spectrum of the ferric complex. The absorbance maxima at 365 and 384 nm are decreased in intensity and the low-spin Fe(III)–BLM complex is partially converted to a high-spin form (g ~4.3), presumably a ferric bleomycin phosphate complex.

Oxidation of Ferrous Bleomycin. The potentiostatic method should be capable of following the oxidation of Fe(II)–BLM in the same way as the reduction of Fe(III)–BLM. However, practical difficulties severely limited this approach. Oxidations occurring at potentials lower than the $E^{\circ\prime}$ for the ferric complex were well-behaved, i.e., they had a characteristic rise time followed by a rapid exponential decay to residual values. As the potential was increased toward the positive range, the residual current increased and became less stable, probably resulting from the generation of new chemical species during the oxidative process. The response time of the potentiostat became longer and an accurate determination of the current–time integral became difficult because of unusually shaped decay curves and failure of the current to return to base line levels.

In an attempt to overcome these difficulties, duplicate samples of iron bleomycin complexes were made, one with the iron in the Fe(II) form and the other in the Fe(III) form, both in 0.05 M Hepes buffer, pH 7.0. Continuous variations of sample mixtures were prepared anaerobically, and the potential range at which the current–time integrals became equal were used as an indicator of the redox potential. The value obtained, 80–120 mV, is in the same range as the reduction potential for Fe(III)–BLM as determined by other methods.

Attempts to monitor the oxidation of Fe(II)–BLM with O_2 by optical means also met with difficulty. A plot of the absorbance vs. the potential was a complicated oxidation titration curve that could not be reasonably fit to a Nernst plot for a single-redox process. Yet, the midpoint potential for the overall reaction was in the same general region as was obtained for the reductive titrations. The use of O_2 to oxidize Fe(II)–BLM most likely leads to the formation of chemical intermediates (Burger et al., 1979b) and a variety of breakdown products.

Discussion

Reduction of Fe(III)–BLM to Fe(II)–BLM has been studied by optical spectroscopy. In electrochemical experiments, excess chemical reducing agents are present, thereby limiting the lower range of reduction to ~0 mV vs. the SHE.

The reduction of Fe(III)–BLM is very slow; more than 15 min is required to achieve equilibrium. Macromolecules such as proteins and nonphysiological reductants also exhibit slow rates of reduction. Mediators which have an important role in maintaining equilibrium between the electrodes or chemical reductants and the macromolecules are often employed in this situation.

In the coulometric–potentiostatic experiments, PMS is an efficient mediator because it reacts rapidly with both the electrode and Fe(III)–BLM and thus greatly increases the rate of reduction of the metal–drug complex. In contrast, the reaction of DCPIP with Fe(III)–BLM is sluggish, and this dye does not function well as a mediator. Since DCPIP reacts quickly with the electrode system, the reduced dye is inefficient in transferring electrons to Fe(III)–BLM. Thus, the use of mediators may complicate the measurements of oxidation–reduction potentials and influence the $E^{\circ\prime}$ value obtained. In the case of iron bleomycin, the same $E^{\circ\prime}$ was obtained by an optical method which does not employ PMS and by a potentiostatic method that uses the dye. This suggests that the electrochemical determination of the redox potential of iron bleomycin in the presence of PMS is valid.

A recent study of the electrochemical properties of bleomycin and tallysomycin and their metallo derivatives, utilizing cyclic voltametry and polarography, has been reported by Dabrowiak & Santillo (1979). These authors detected two reduction processes for metal-free BLM, one at −1.22 V assigned to the two-electron reduction of the 4-aminopyrimidine moiety and the second at −1.48 V assigned to the two-electron reduction of the bithiazole moiety. For the iron complex, no reduction of Fe(III) to Fe(II) could be demonstrated either in the presence of phosphate buffer or in unbuffered solutions. This could be due to several reasons. With polarography, the potential window being viewed by the dropping mercury electrode is, for the most part, much more negative than the oxidation–reduction potential of iron bleomycin as determined in our present study. Therefore, Fe(III)–BLM may have been reduced from the outset of the experiments. In addition, the method of preparation of Fe(III)–BLM greatly affects the coordination of the iron and the resulting stability of the complex. Phosphate has been shown, for example, to interact with Fe(III)–BLM (Burger et al., 1979b). The use of unbuffered solutions also leads to difficulties because the reduction process may demonstrate strong pH dependence. Furthermore, the absence of mediators in the experiment may have altered the rate of reduction. Without PMS, the potential scan rate with a voltameter may have been too rapid to observe the reduction of iron. If cyclic voltametry or polarography is utilized without a mediator, the redox couple may appear irreversible or be shifted in the direction of the cathodic potentials so as to overcome the energy barrier for reduction.

Although Fe(III)–BLM is altered in phosphate buffer, the complex is maintained in Hepes which is a suitable buffer system for the determination of the redox potential. By maintaining the pH at neutrality and not competing for iron, Hepes stabilizes the bound iron in both oxidation states, thereby preventing the formation of other chemical species of iron.

An $E^{\circ\prime}$ of 129 mV for iron bleomycin places it in the range of known Fe(III/Fe(II) redox couples found in biological systems. These vary from +400 mV for some cytochromes to −400 mV for nonheme multiiron centers (Loach, 1968). Since these values are, in general, lower than that for the aqueous Fe(III)/Fe(II) couple, a general electrostatic stabilization effect is seen for most iron complexes in biological systems (Fuhrhop, 1974). For example, the aqueous Fe(III)/Fe(II) couple is +530 mV. Aqueous Fe(III) would then require ~10 kcal/mol less energy to undergo reduction than does Fe(III)–BLM.

The similarity of the redox potential for iron bleomycin to those of hemoglobin ($E^{\circ\prime}$ = 144 mV) (Taylor & Hastings, 1939) and tryptophan oxygenase ($E^{\circ\prime}$ = 105 mV) (Feigelson & Brady, 1974) suggests that the iron environment in the drug complex is suitable for O_2 binding and/or activation.

The degradation of DNA by Fe(II)–BLM results in the formation of Fe(III)–BLM. If the Fe(III) remains bound to the drug, then reducing agents with potentials more negative than +129 mV will reduce Fe(III)–BLM to Fe(II)–BLM. Relatively strong reductants such as NADH ($E^{\circ\prime}$ = −318 mV), ascorbate ($E^{\circ\prime}$ = −58 mV), glutathione ($E^{\circ\prime}$ = −230 mV), cysteine ($E^{\circ\prime}$ = −340 mV), or superoxide ($E^{\circ\prime}$ = −330 mV) (Clark, 1960; Wood, 1974) have sufficiently low redox potentials to drive the reduction, while weak reductants such as H_2O_2 ($E^{\circ\prime}$ = 270 mV) (George, 1965) do not. With the regeneration of Fe(II)–BLM from Fe(III)–BLM, the catalytic cleavage cycle would proceed until either the reducing equivalents or the DNA become depleted.

Acknowledgments

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References

Antholine, W. E., & Petering, D. H. (1979) *Biochem. Biophys. Res. Commun. 90*, 384–389.

Burger, R. M., Peisach, J., Blumberg, W. E., & Horwitz, S. B. (1979a) *J. Biol. Chem. 254*, 10906–10912.

Burger, R. M., Horwitz, S. B., Peisach, J., & Wittenberg, J. B. (1979b) *J. Biol. Chem. 254*, 12299–12302.

Burger, R. M., Adler, A. D., Horwitz, S. B., Mims, W. B., & Peisach, J. (1981) *Biochemistry 20*, 1701–1704.

Clark, W. M. (1960) in *Oxidation Reduction Potential of Organic Systems*, pp 405–495, Williams and Wilkens, Baltimore, MD.

Dabrowiak, J. C., Greenway, F. T., Longo, W. E., Husen, M. V., & Crooke, S. T. (1978) *Biochim. Biophys. Acta 517*, 517–526.

Dabrowiak, J. C., & Santillo, F. S. (1979) *J. Electrochem. Soc. 126*, 2091–2095.

Dickens, F., & McIlwain, H. (1938) *Biochem. J. 32*, 1615.

Feigelson P., & Brady, F. O. (1974) in *Molecular Mechanisms of Oxygen Activation* (Hayaishi, O., Ed.) pp 87–133, Academic Press, New York.

Fuhrhop, J. H. (1974) *Struct. Bonding (Berlin) 18*, 1–67.

George, P. (1965) in *Oxidases and Related Redox Systems* (King, T. E., Mason, H. S., & Morrison, M., Eds.) pp 3–36, Wiley, New York.

Gibbs, H. D., Cohen, B., & Cannan, R. K. (1925) *Public Health Rep. 40*, 649 (Reprint No. 1001).

Iitaka, Y., Nakamura, H., Nakatani, T., Muraoka, Y., Fujii, A., Takita, T., & Umezawa, H. (1978) *J. Antibiot. 31*, 1070–1072.

Ishida, R., & Takahashi, T. (1975) *Biochem. Biophys. Res. Commun. 66*, 1432–1438.

Loach, P. A. (1968) in *Handbook of Biochemistry* (Sober, H. A., Ed.) pp J27–J34, Chemical Rubber Co., Cleveland, OH.

Onishi, T., Iwata, H., & Takagi, Y. (1975) *J. Biochem. (Tokyo) 77*, 745–752.

Oppenheimer, N. J., Rodriguez, L. O., & Hecht, S. M. (1979)

Proc. Natl. Acad. Sci. U.S.A. 76, 5616–5620.
Sausville, E. A., Peisach, J., & Horwitz, S. B. (1976) *Biochem. Biophys. Res. Commun. 73*, 814–822.
Sausville, E. A., Stein, R. W., Peisach, J., & Horwitz, S. B. (1978) *Biochemistry 17*, 2746–2754.
Takita, T., Muraoka, Y., Nakatani, T., Fujii, A., Umezawa, Y., Naganawa, H., & Umezawa, H. (1978) *J. Antibiot. 31*, 801–804.
Taylor, J. F., & Hastings, A. B. (1939) *J. Biol. Chem. 131*, 649–662.
Umezawa, H. (1975) *Antibiotics (N.Y.) 3*, 21–33.
Wood, P. M. (1974) *FEBS Lett. 44*, 22–25.

Nonspecific Cleavage of ϕX174 RFI Deoxyribonucleic Acid by Bleomycin†

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ABSTRACT: The covalently closed circular duplex deoxyribonucleic acid (DNA) of ϕX174 underwent progressive conversion to nicked and linear DNA with increasing bleomycin/ϕX174 RFI DNA molecule ratios. The formation of linear DNA (a double-strand break) occurred under limited reaction conditions as low as an average of 0.2 single-strand break/ϕX174 RFI DNA molecule. As bleomycin-produced linear DNA was further fragmented by bleomycin, a broad distribution of DNA fragments without notable concentrations of unique size was formed. Restriction enzymes *Pst*I and *Sst*II did not generate discrete fragments from bleomycin-produced full-length linear ϕX174 DNA, nor did bleomycin cleavage generate discrete fragments from *Hpa*II or *Pst*I digests of ϕX174 RFI. These findings suggest that bleomycin does not act at a few specific sites on ϕX174 RFI DNA. The single-strand nick appeared to be the preferred site for bleomycin action for a second cleavage in a ϕX174 molecule.

Bleomycin is a complex, low molecular weight, glycopeptide antibiotic used clinically as an antitumor agent, primarily against a variety of solid tumors (Umezawa, 1975). The precise mechanism by which bleomycin causes cell death is unknown but is thought to be mediated by its interaction with DNA. Bleomycin is known to produce a variety of different effects on DNA, including loss of free base (Haidle et al., 1972; Muller & Zahn, 1976), production of alkali-sensitive sites (Ross & Moses, 1978), and single- and double-strand breaks (Suzuki et al., 1969; Haidle, 1971; Umezawa et al., 1973). At low bleomycin concentrations, thymine is selectively released (Muller & Zahn, 1976), and thymine 3′ to guanine is preferred [Maxam & Gilbert (1977), reported in Poon et al. (1977)]. At high concentrations, all four bases are released (Haidle et al., 1972). Under the appropriate reaction conditions, both single- and double-strand breaks in DNA are produced by bleomycin action. The breaks formed by bleomycin action are not simple phosphodiester bond nicks, because they appear to bear either 3′- or 5′-phosphoryls (Kuo & Haidle, 1973; Shapiro & Chargaff, 1964) and contain gaps where thymine and other bases have been removed (Poon et al., 1977; Ishida & Takahashi, 1976). At high bleomycin/nucleotide ratios, breaks occur preferentially 3′ to GT and GC sequences and to a lesser extent at other sequences containing thymine (D'Andrea & Haseltine, 1978; Takeshita et al., 1978).

It has been suggested that at low bleomycin concentrations double-strand breaks occur at specific sites in the genome of the closed circular bacteriophage PM2 (Lloyd et al., 1978a). Thus far, 11 apparent regions of BLM preference have been identified and mapped on the PM2 genome (Lloyd et al., 1978a,b). In view of the site preferences of bleomycin-produced double-strand breaks, we thought it of interest to analyze the preferred sites for common nucleotide sequences. We have examined bleomycin action on ϕX174 RFI DNA, a closed circular duplex for which the entire base sequence has been published (Sanger et al., 1977). To facilitate sequence analysis, we decided to investigate bleomycin action leading to the first DNA double-strand break. In this report, we demonstrate the lack of site-specific double-strand break formation on the ϕX174 RFI genome under limited reaction conditions. In accord with previously published results, we find that double-strand breaks occur more rapidly than would be predicted by random accumulation of single-strand breaks (Lloyd et al., 1978a; Povrik et al., 1977).

Experimental Procedures

Materials. Blenoxane, clinical bleomycin sulfate, was obtained from Bristol Laboratories. Agarose, acrylamide (recrystallized), *N,N′*-methylenebis(acrylamide), *N,N,N,N′*-tetramethylethylenediamine, and ammonium persulfate were products of Bio-Rad Laboratories. Nitrocellulose filters (BA 85) were obtained from Schleicher & Schüll. All restriction enzymes were obtained from Bethesda Research Labs. [γ-^{32}P]Adenosine triphosphate was obtained from Amersham/Searle.

Preparation of ϕX174 RFI DNA and [^{3}H]ϕX174 RFI DNA. The procedure employed in isolation of the covalently closed circular form of ϕX174 was that of Schekman et al. (1971), with the modifications of Ross & Moses (1978). Following the final precipitation of the supercoiled fraction from cesium chloride–ethidium bromide gradients, the DNA

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