Vol. 92, No. 1, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1980 Pages 197-205

BLEOMYCIN-A_2 COMPLEXES WITH POLY(dA-dT): A PROTON NUCLEAR MAGNETIC RESONANCE STUDY OF THE NONEXCHANGEABLE HYDROGENS

Douglas M. Chen,* Ted T. Sakai,* Jerry D. Glickson* and Dinshaw J. Patel†

*Molecular Pharmacology and Biophysics Program of the Comprehensive Cancer Center and Department of Biochemistry, University of Alabama in Birmingham, Birmingham, AL 35294, and †Bell Laboratories, Murray Hill, New Jersey 07974, USA

Received November 30,1979

SUMMARY: Binding of bleomycin-A_2 (Bleo-A_2) to poly(dA-dT) in 100 mM sodium phosphate (pH 6.8) in D_2O has been investigated by ^1H NMR spectroscopy at 270 and 360 MHz. Significant spectral perturbations were observed only when the nucleic acid was in the duplex state. Of the Bleo-A_2 resonances, the two bithiazole peaks exhibited the largest spectral shifts and line broadening effects. The high field shift of these resonances was very small near room temperature and reached a maximum of about 0.27 ppm just below the thermal denaturation temperature of the nucleic acid ($T_m = 60 \pm 1^oC$). The temperature dependence of spectral perturbations may be accounted for by the formation of at least two types of Bleo-A_2 complexes with the polynucleotide. Other perturbed resonances of bleomycin are the S-CH_3 and S-CH_2 of the terminal amine, the CH_2-N resonance of the bithiazole residue, and the $CH(CH_3)CO$ of the methyl-valeric acid residue. The significantly perturbed resonances of the nucleic acid originate from the A(H-2), A(H-8), T(H-6) and one of the H-2' hydrogens. Binding of the C-terminal tripeptide fragment of Bleo-A_2 to poly(dA-dT) is accompanied by selective broadening of the bithiazole group. These experiments have identified potential loci of interaction on the Bleo-A_2 and poly(dA-dT) molecules.

Bleomycin-A_2 (Bleo-A_2; Figure 1) is one of a mixture of closely related glycopeptide antibiotics (the bleomycins) employed in cancer chemotherapy and tumor scanning (1-4). These compounds cause degradation of DNA *in vivo* and *in vitro* (5). Although it has not yet been definitively proven that DNA degradation is responsible for the pharmacological activity of these agents, some mode of association with DNA appears to be implicated in the *in vivo* reaction (2-4). Characterization of the structure of bleomycin complexes with nucleic acids is, therefore, a crucial step toward elucidating the mode of pharmacological action of these agents.

The interaction of the bleomycins and closely related antibiotics with DNA and synthetic polynucleotides has been investigated by a variety of methods (6-12). Recently, Chien *et al.* (11) have reported some preliminary proton nuclear magnetic resonance (^1H NMR) experiments on Bleo-A_2 binding to calf thymus DNA. Selective broadening of Bleo-A_2 resonances implicated the bithiazole and dimethylsulfonium groups as the most tightly bound moieties of the drug. No information was obtained on the receptor DNA. The present inves-

0006-291X/80/010197-09$01.00/0

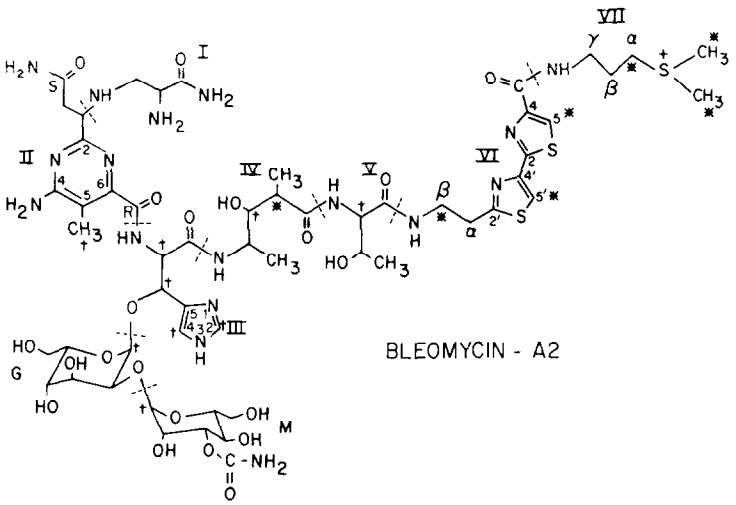

Figure 1: Structure of Bleo-A_2 (free base). Boundaries between residues (denoted by Roman numerals), M (mannose) and G (gulose) are indicated by broken lines. Hydrogens whose resonances are perturbed on binding to poly(dA-dT) (*) and those which are not (†) have been appropriately labeled.

tigation represents an extension of these experiments. At the higher field employed in these experiments it is possible to monitor a wider range of Bleo-A_2 resonances and to observe spectral shifts that were not previously reported. Because of its rigid and complex structure, DNA yields ^{1}H NMR spectra which are too broad and too complicated to analyze by normal high resolution procedures; therefore, we have also chosen to replace native DNA by a synthetic analogue, poly(dA-dT), whose relatively simple spectrum yields considerable information on the structure and dynamics of complexes of this polynucleotide with various drugs (13).

EXPERIMENTAL

Poly(dA-dT). Poly(dA-dT) (S_{20} = 9.5) was obtained from Collaborative Research, Inc. (Cambridge, Mass.) and purified as described previously (13). Concentrations were determined using a molar absorptivity per nucleotide of 6.60×10^3 cm^{-1} M^{-1} at 262 nm. [A-8-D]Poly(dA-dT) was prepared by heating poly(dA-dT) in D_2O (70-75°, 20-24 hrs; > 95% D at A-8).

Bleomycin-A_2. Bleomycin-A_2 was purified from commercial Blenoxane (a generous gift of Drs. W.T. Bradner and S.T. Crooke, Bristol Laboratories, Syracuse, N.Y.) by ion-exchange chromatography on carboxymethyl Sephadex C-25 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) employing a linear gradient of triethylammonium bicarbonate and converted to the phosphate salt as described (14). By the procedure of Yoda and Hokin (15) it was demonstrated that stoichiometric amounts of phosphate were present. Concentrations of Bleo-A_2 were determined using a molar absorptivity of 1.5×10^4 cm^{-1} M^{-1} at 292 nm.

Tripeptide-S, the C-terminal fragment of Bleo-A_2 (residues V-VII), was obtained by partial hydrolysis of the antibiotic (3).

NMR Spectra. All NMR experiments were carried out in deuterium oxide containing 100 mM sodium phosphate - 1 mM EDTA, pH_m = 6.8 (pH_1 meter reading

uncorrected for deuterium isotope effects). High resolution H NMR spectra were measured on Bruker HX-270 and HX-360 spectrometers operated in the pulse Fourier transform mode. Samples were contained in 5 mm o.d. cylindrical microcells (Wilmad Glass Company, Buena, N.J.). The temperature was determined to $\pm$ 1°C from the separation of ethylene glycol resonances. Chemical shifts are reported relative to internal $(CH_3)_3Si(CD_2)_2CO_2Na$ (Stohler Isotope Chemicals, Waltham, Mass.).

RESULTS AND DISCUSSION

General Considerations. The resonances of Bleo-A_2 (16) and poly(dA-dT) (13) have been previously assigned. The observation of a single resonance for each chemically distinct hydrogen of the drug and nucleic acid at all temperatures (0-75°C) and all nucleic acid-phosphate to drug ratios (P/D = 5-70) examined in this study indicates that under these conditions all the interaction and conformational changes are at least moderately fast on the NMR time scale. The reversibility of all thermally induced perturbations of resonances of Bleo-A_2 and of poly(dA-dT) was confirmed by performing the experiments in two stages -- first increasing the temperature in small increments and then decreasing it. For each resonance, both sets of points fell on the same curve (see below).

Poly(dA-dT) Resonances. Figure 2 shows the temperature dependence of the chemical shifts of some of the poly(dA-dT) resonances in the presence and absence of Bleo-A_2. The helix-coil transition, whose midpoint (T_m) occurs at 60 $\pm$ 1°C, produces a very sharp shift of all the base resonances from high to low field as a result of the elimination of ring current shifts associated with base stacking interactions (Figure 2). The sugar resonances exhibit high field or low field shifts depending on their orientation in the helix relative to the ring-current fields of the stacked bases (13). All the thermal profiles indicate a highly cooperative monophasic helix-coil transition. Binding of Bleo-A_2 has no significant effect on the T_m, but does perturb the A(H-2), A(H-8), T(H-6) (Fig. 2) and one of the H-2' resonances (not shown). The T(CH_3), A(H-1'), T(H-1'), A(H-3') and T(H-3') resonances are not significantly perturbed by drug binding. All the spectral perturbations associated with Bleo-A_2 binding occur in the helical state (below T_m) indicating minimal interaction between the drug and the random-coil polymer. The drug-induced perturbations of the T(H-6) (Fig. 2) and H-2' resonances increase with decreasing temperature, whereas the A(H-2) and A(H-8) resonances (Fig. 2) peaks exhibit more uniform spectral displacements. In all cases the spectral shift associated with drug binding is in a direction which diminishes the spectral shift associated with the helix-coil transition, i.e., the A(H-2), A(H-8) and T(H-6) resonances are shifted to low

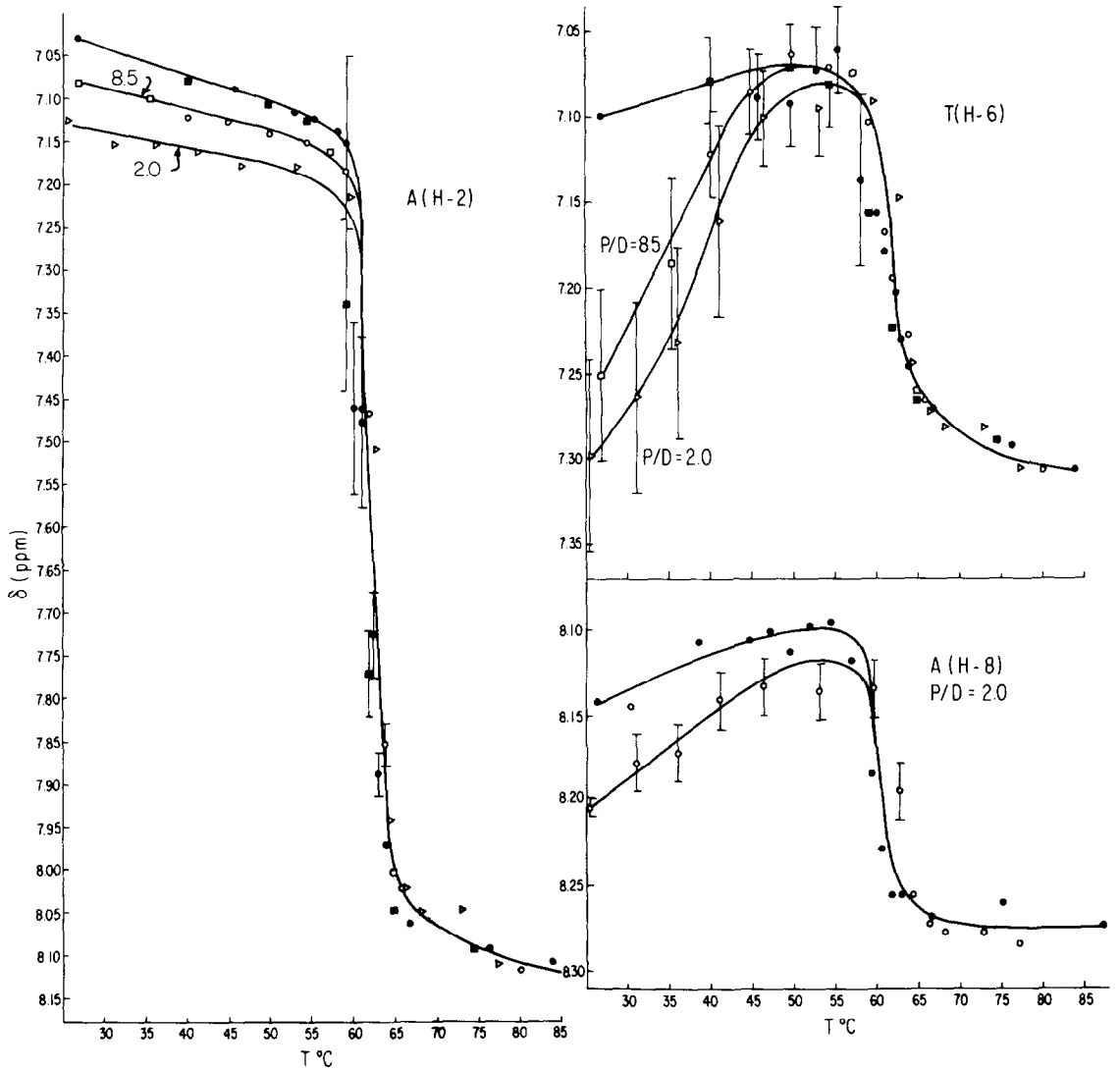

Figure 2: Temperature dependence of the chemical shifts of the A(H-2), A(H-8) and T(H-6) hydrogens of poly(dA-dT). Solid symbols (● , ■) denote data points in the absence of Bleo-A_2; open symbols (○ , □ , △) denote data points in the presence of Bleo-A_2 at the indicated P/D. Circles (○ , ●) correspond to points measured on the increasing temperature phase of the experiment; squares (□ , ■) correspond to the decreasing temperature phase. The control (no Bleo) and the P/D = 8.5 experiments were performed with 17 mM poly(dA-dT) deuterated at the A-8 position. The P/D = 2.0 study and the control for the A(H-8) measurements were performed with 15 mM poly(dA-dT) which had not been isotopically modified at the A-8 position. All measurements were made at 270 MHz except for the A(H-8) and P/D = 2.0 experiments which were performed at 360 MHz.

field, whereas the H-2' peak is shifted to high field. The shifts are all very small, being no larger than 0.2 ppm.

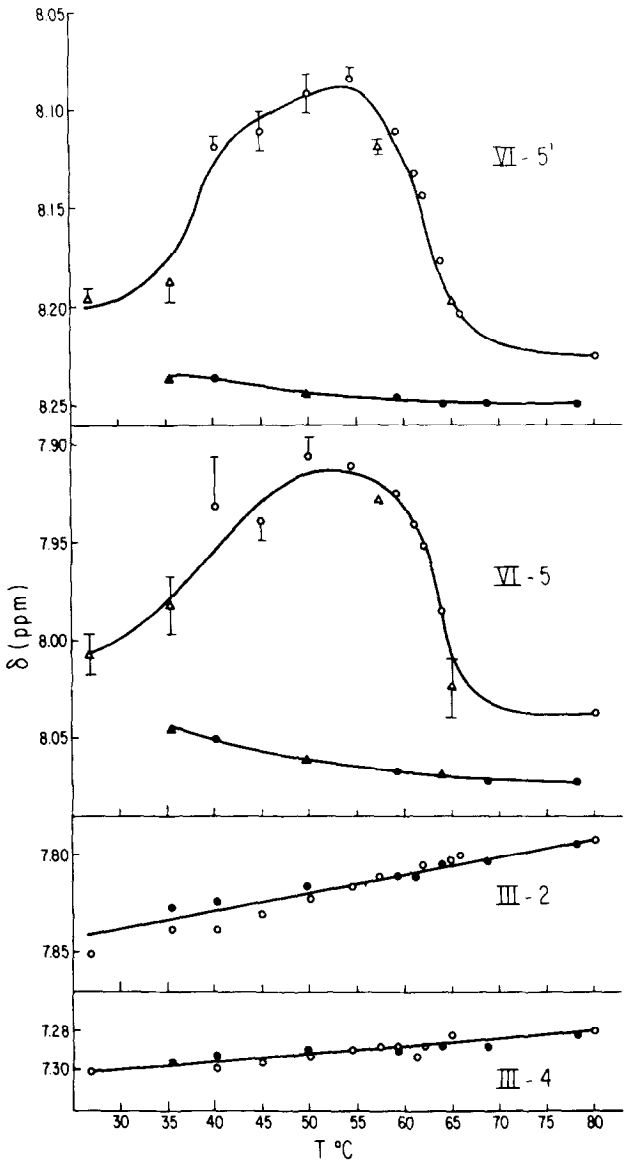

Figure 3: Temperature dependence of the chemical shifts of resonances (270 MHz) originating from the aromatic CH hydrogens of Bleo-A_2. The symbols and experimental conditions are the same as for the A(H-2) study in Figure 2 except that the control experiments denoted by solid figures correspond to free Bleo-A_2 (5 mM). The open symbols (○, □) correspond to the P/D = 8.5 samples described in Figure 2.

Bleo-A_2 Resonances. The thermal profiles of the chemical shifts of some of the Bleo-A_2 resonances appear in Figures 3 and 4. Consistent with the poly(dA-dT) data (see above), significant perturbations are observed only at temperatures at which the polynucleotide is helical. The very weak perturbations

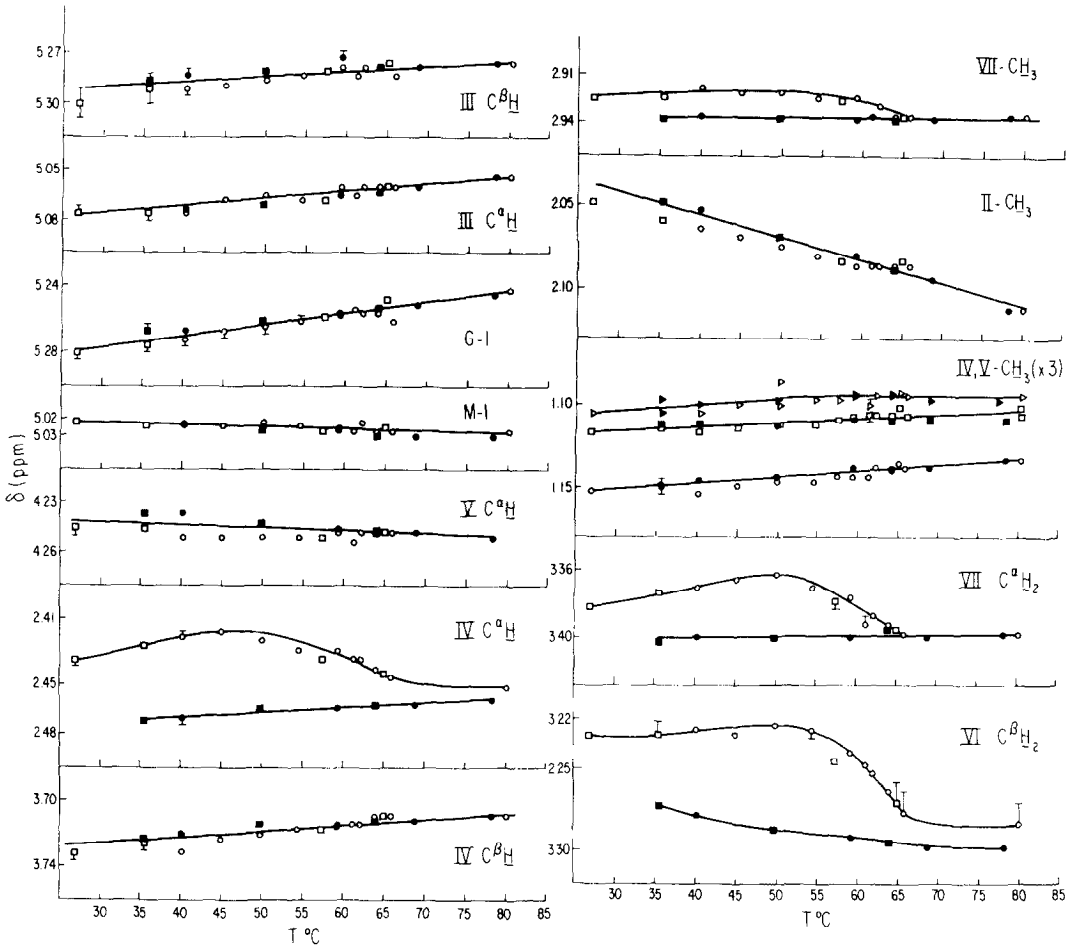

Figure 4: Temperature dependence of the chemical shifts of some of the aliphatic CH hydrogens of Bleo-A$_2$. The notation and conditions are the same as in Figure 3.

of the VI-C$^{\beta}$H$_2$ (Fig. 4) and VI-5 and VI-5' (Fig. 3) resonances at higher temperatures may indicate some weak residual interactions with the random-coil polymer.

Perturbed and unperturbed resonances have been appropriately indicated in Fig. 1. The spectral perturbations, some of which (but not all of which) must originate from direct interaction with poly(dA-dT), are localized on residues IV-VII.

The central role played by the bithiazole group in the binding of Bleo-A$_2$ to poly(dA-dT) is indicated by the magnitude of the spectral shift exhibited by resonances VI-5 and VI-5' (Fig. 3), by far the largest of all the Bleo-A$_2$ peaks, and by preferential broadening of these resonances, the only such effect exhibited by any of the Bleo-A$_2$ peaks. Since the extent of broadening did not vary substantially with field (270 and 360 MHz), the origin of this effect is

probably dipolar, and it may be concluded that the bithiazole group is probably the most tightly bound moiety of Bleo-A_2.

The displacement of the bithiazole resonances is maximal near 50°C and diminishes at lower temperatures (Fig. 3). That this diminution is probably not caused by dissociation of the Bleo-A_2 is indicated by the retention of substantial shifts of other resonances of the drug and nucleic acid at lower temperatures; indeed, the perturbations of the T(H-6) (Fig. 2) and H-2' peaks is augmented at lower temperatures. The temperature dependence of the bithiazole shifts therefore argues for the existence of at least two classes of Bleo-A_2/poly(dA-dT) complexes -- a type-I complex preferred near 50°C which is associated with a significant high field shift of the bithiazole resonances and a type-II complex preferred at lower temperatures which is associated with substantially smaller displacements of these resonances. The maximum shift of the bithiazole resonances, 0.27 ppm, was determined by least squares fitting of a plot of chemical shift at 50°C vs. P/D. This shift is in the same direction but of considerably lower magnitude than shifts exhibited by resonances of classical intercalating agents (13).

The spectrum of tripeptide S in the presence of poly(dA-dT) shows a selective broadening of the two low field bithiazole resonances, similar in magnitude to the broadening of these resonances in the spectrum of Bleo-A_2. These data indicate, in agreement with fluorescence experiments of Chien et al. (12) that tripeptide S binds to the nucleic acid. However, in the absence of additional analysis of this interaction, which awaits assignment of the remaining resonances of the tripeptide, it cannot be determined if this fragment binds in a manner similar to the parent antibiotic, except that in both molecules the bithiazole group is probably the most tightly bound moiety.

CONCLUSIONS

The small magnitude of the spectral shifts observed for both the drug and nucleic acid prevents one from drawing definitive conclusions about the modes of binding Bleo-A_2 to poly(dA-dT). It is, however, clear that there are at least two types of complexes formed. The type-I complex may involve some mode of insertion of the bithiazole group into the poly(dA-dT) double helix, whereas the type-II complex probably does not. It is perhaps noteworthy that any insertion of the bithiazole group which might occur is observed just below the helix-coil transition, i.e., in a temperature range in which fluctuations away from the helical state involving "kinks" or perhaps other structures are expected to be favored (17-19). Such fluctuations may facilitate type-I binding. A classical intercalation mechanism for type-I complexes, which has been suggested by Povirk (12), appears unlikely in view of the small magnitude

of the spectral shifts and the viscosity data of Chien et al. (11). These shifts, however, depend on the detailed orientation of the bithiazole group relative to the planar bases and on the ring current fields of the interacting aromatic rings. In view of the very similar thermal profiles of both of the bithiazole proton resonances, it appears likely that they experience very similar environments in the type I and perhaps also type II complexes. The absence of any effect of drug binding on the T(CH_3) resonances suggests that whatever type of interaction is occurring is due to binding in the minor groove of poly(dA-dT), since the methyl groups face into the major groove. Additional studies on the nature of these interactions are in progress.

The experiments described in this study confirm the bifunctional nature of the bleomycin molecule, whose N-terminal end is the principal moiety involved in binding of polyvalent metals (20-22) and whose C-terminus is the site of interaction with nucleic acids (11).

ACKNOWLEDGEMENTS

Studies at the University of Alabama were supported by Public Health Service Grants CA-13148 (John R. Durant and J.D.G.) and CA-24411 (J.D.G.), and by grant IN-66Q (D.M.C.) and Faculty Research Award FRA-162 (J.D.G.) from the American Cancer Society. Experiments employing the Bruker HX-270 spectrometer were conducted at the Florida State University NMR Facility. The authors are indebted to Ms. Lita Canuel for technical assistance.

REFERENCES

1. See articles in: Bleomycin. Current Status and New Developments, Carter, S.K. Crooke, S.T., and Umezawa, H., Eds., 1978, Academic Press, New York.
2. Umezawa, H. (1973) Biomedicine 18, 459-474.
3. Umezawa, H. (1974) Fed. Proc. 33, 2296-2302.
4. Crooke, S.T., and Bradner, W.T. (1976) J. Med. 7, 333-428.
5. See Sausville, E.A., Peisach, J., Horwitz, S.B. (1978) Biochemistry 17, 2740-2746 and references therein.
6. Pietsch, P. (1973) Biotech. Bioeng. 15, 1039-1044.
7. Suzuki, H., Nagai, K., Akutsu, E., Yamaki, H., Tanaka, N. and Umezawa, H. (1970) J. Antibiot. 23, 473-480.
8. Muller, W.E.G., Yamazaki, Z.I., Breter, H.J. and Zahn, R.K. (1972) Eur. J. Biochem. 31, 518-525.
9. Krueger, W.C., Pschigoda, M. and Reusser, F. (1973) J. Antibiot. 26, 424-428.
10. Pietsch, P. and Garrett, H. (1968) Nature 219, 488-489.
11. Chien, M., Grollman, A.P. and Horwitz, S.B. (1977) Biochemistry 16, 3641-3647.
12. Povirk, L.F., Hogan, M. and Dattagupta, N. (1979) Biochemistry 18, 96-101.
13. Patel, D.J. (1979) Acc. Chem. Res. 12, 118-125.
14. Sakai, T.T. and Riordan, J.M. (1979), J. Chromatog. 178, 302-306.
15. Yoda, A. and Hoken, L.E. (1970) Biochem. Biophys. Res. Commun. 40, 880-884.
16. Chen, D.M. Hawkins, B.L. and Glickson, J.D. (1977) Biochemistry 16, 2731-2738.

17. Gabbay, E.J. (1977) Bioorganic Chem., Vol. 3, Macromolecular and Micro-Molecular Systems, pp. 33-69, Academic Press, New York.
18. Sobell, H.M., Reddy, B.S., Bhanjari, K.K., Jain, S.C., Sakore, T.D. and Seshadri, T.T. (1978) Cold Springs Harbor Symposia Quant. Biol. 42, 87-102.
19. Sobell, H.M., Lozansky, E.D. and Lessen (1979), ibid 43, 11-19.
20. Dabrowiak, J.C., Greenaway, F.T. and Grulich, R. (1978) Biochemistry 17, 4090-4096.
21. Cass, A.E.G., Galdes, A.G.H., Hill, O. and McClelland (1978) FEBS Lett. 89, 187-190.
22. Gupta, R.J., Ferretti, J.A. and Caspary, W.J. (1979) Biochem. Biophys. Res. Commun. 89, 534-541.