Biochemistry **1988**, *27*, 915–923

Guinier, A. (1939) *Ann. Phys. (Paris) 12*, 161.
Herzberg, O., & James, M. N. G. (1985) *Nature (London) 313*, 653.
Klee, C. B., & Vannaman, T. C. (1982) *Adv. Protein Chem. 35*, 213.
McUbbin, W. D., Oikawa, K., & Kay, C. M. (1986) *FEBS Lett. 195*, 17.
Means, A. R., & Dedman, J. R. (1980) *Nature (London) 258*, 73.
Moews, P. C., & Kretsinger, R. H. (1975) *J. Mol. Biol. 91*, 201.
Moore, P. B. (1980) *J. Appl. Crystallogr. 13*, 168.
Porod, G. (1951) *Kolloid-Z. 124*, 83.
Potter, J. D., & Gergely, J. (1975) *J. Biol. Chem. 250*, 4628.
Seaton, B. A., Head, J. F., Engelman, D. M., & Richards, F. M. (1985) *Biochemistry 24*, 6740.
Sundaralingam, M., Bergstrom, R., Strasburg, G., Rao, S. T., Roychowdhury, P., Greaser, M., & Wang, B. C. (1985a) *Science (Washington, D.C.) 227*, 945.
Sundaralingam, M., Drendel, W., & Greaser, M. (1985b) *Proc. Natl. Acad. Sci. U.S.A. 82*, 944.
Szebenyi, D. M. E., Obendorf, S. K., & Moffat, K. (1987) *Nature (London) 294*, 327.
Wang, C. K., & Cheung, H. C. (1985) *Biochemistry 24*, 3365.
Wang, C.-L. A., Zhan, Q., Tao, T., & Gergely, J. (1986) *Biophys. J. 49*, 48a.
Watterson, D. M., Sharief, F., & Vanaman, T. C. (1980) *J. Biol. Chem. 255*, 962.
Wery, J. P., Dideberg, O., Charlier, P., & Gerday, C. (1985) *FEBS Lett. 182*, 103.
Wilkinson, J. M. (1985) *Biochim. Biophys. Acta 359*, 379.
Zaccai, G., & Jacrot, B. (1983) *Annu. Rev. Biophys. 12*, 139.

---

^{1}H and ^{31}P NMR Investigations of Actinomycin D Binding Selectivity with Oligodeoxyribonucleotides Containing Multiple Adjacent d(GC) Sites[†]

Elwood V. Scott,[‡] Robert L. Jones,[§] Debra L. Banville,[‡] Gerald Zon,[∥,⊥] Luigi G. Marzilli,[*,‡] and W. David Wilson[*,§]

Department of Chemistry, Georgia State University, Atlanta, Georgia 30303, Department of Chemistry, Emory University, Atlanta, Georgia 30322, and Molecular Pharmacology Laboratory, Division of Biochemistry and Biophysics, Food and Drug Administration, Bethesda, Maryland 20892

Received March 17, 1987; Revised Manuscript Received September 22, 1987

ABSTRACT: Imino proton and ^{31}P NMR studies were conducted on the binding of actinomycin D (ActD) to self-complementary oligodeoxyribonucleotides with adjacent 5′-GC-3′ sites. ActD showed very high specificity for binding to GC sites regardless of oligomer length and surrounding sequence. For a first class of duplexes with a central GCGC sequence, a mixture of 1:1 complexes was observed due to the two different orientations of the ActD phenoxazone ring system. Analysis of ^{1}H chemical shifts suggested that the favored 1:1 complex had the benzenoid side of the phenoxazone ring over the G base in the central base pair of the GCGC sequence. This is the first case in which an unsymmetrical intercalator has been shown to bind to DNA in both possible orientations. A unique 2:1 complex, with significantly different ^{1}H and ^{31}P chemical shifts relative to those of the 1:1 complexes, was formed with these same oligomers, again with the benzenoid side of the ActD molecule over the G base of the central GC base pair. There is considerable anticooperativity to binding of the second ActD in a GCGC sequence. In titrations of oligomers with the GCGC sequence, only the two 1:1 complexes are found up to ratios of one ActD per oligomer. Increasing the ActD concentration, however, resulted in stoichiometric formation of the unique 2:1 adduct. Spectrophotometric binding studies indicated that the apparent binding equilibrium constant for a GC site adjacent to a bound site is reduced by approximately a factor of 20 relative to the ActD binding constant to an isolated GC site. Both upfield and downfield shifts were seen for imino proton signals for base pairs adjacent to ActD binding sites. This suggests that ActD has considerable long-range effects on oligomer conformation. Anticooperativity was also seen in NMR studies with a second class of oligomers containing alternating GC sequences longer than GCGC. It was found that in any three consecutive GC binding sites only two ActD can be bound. Anticooperativity can, thus, define the apparent number of base pairs in the binding site of an intercalator. Binding results with poly[d(G-C)]·poly[d(G-C)] were similar to those obtained with an oligomer containing four consecutive GC sites.

Actinomycin D (ActD)[1] was one of the first antibiotics whose interaction with DNA was investigated in depth [reviewed in Waring (1981)], and its DNA complex has been used as a model for DNA–protein interactions (Sobell, 1973). Müller and Crothers (1968) in a detailed hydrodynamic, kinetic, and thermodynamic study on several ActD analogues proposed that this molecule bound to DNA by intercalation,

---

[†] This work was supported by NSF Grant DBM-8603566 to W.D.W. and NIH Grant GM 29222 to L.G.M.

[*] Author to whom correspondence should be addressed.

[‡] Emory University.

[§] Georgia State University.

[∥] Food and Drug Administration.

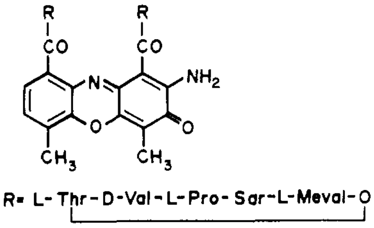
[⊥] Present address: Applied Biosystems, 850 Lincoln Centre Drive, Foster City, CA 94404.

[1] Abbreviations: ActD, actinomycin D; DNA, deoxyribonucleic acid; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); poly[d(G-C)]$_2$, poly[d(G-C)]·poly[d(G-C)]; EDTA, ethylenediaminetetraacetic acid; 2D, two dimensional; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; TSP, 3-(trimethylsilyl)propionic acid. Interchain base pairs are indicated with a dot, e.g., G·C, while intrachain sequences are indicated without a dot or dash, e.g., GC or GCGC; all sequences are written in the 5′ → 3′ direction.

0006-2960/88/0427-0915$01.50/0

d(1-2-3-4-5-6-7-7-6-5-4-3-2-1)
G-C-G-C
T-G-C-G-C-A
A-T-G-C-G-C-A-T
T-A-T-G-C-G-C-A-T-A
T-A-T-A-T-G-C-G-C-A-T-A-T-A

T-A-T-G-C-G-C-G-C-A-T-A
T-A-T-G-C-G-C-G-C-G-C-A-T-A
$d(G\text{-}C\text{-}G\text{-}C)_n$



ACTINOMYCIN D

FIGURE 1: The top five oligomers (group I) all contain the GCGC sequence. The next two oligomers (group II) contain one and two additional GC sites, and at the bottom is poly$[d(G\text{-}C)]_2$, $d(G\text{-}C\text{-}G\text{-}C)_n$. The structure of ActD is shown at the bottom of the figure.

but with very slow and complex kinetics. This evidence for intercalation was supported by results which indicated that ActD could unwind superhelical DNA (Waring, 1970). Sobell and co-workers [reviewed in Sobell (1973)] were able to solve the X-ray structure of a crystalline adduct containing ActD and deoxyguanosine in a 1:2 ratio. In this structure the two cyclic peptides of ActD (Figure 1) were related by 2-fold symmetry and formed strong, specific hydrogen bonds to the 2-NH_2 groups and N-3 ring nitrogens of the guanine bases that were stacked on alternate sides of the phenoxazone ring system of ActD (Sobell, 1973). In model building experiments, Sobell and co-workers (Sobell, 1973) added deoxycytidine to base pair with the G residues in the crystal structure and were able to build stereochemically reasonable structures for ActD intercalated into B-form DNA. The hydrogen bonds to G in the model explain the G binding specificity of the antibiotic and predict a strong GC[1] sequence binding specificity for ActD (Sobell, 1973).

The model for intercalation at GC sites was supported by a series of NMR experiments with di- and oligonucleotides by Krugh, Patel, and their co-workers [reviewed in Krugh and Nuss (1979)]. Recently, the model has been supported by more detailed 1D and 2D proton NMR experiments (Reid et al., 1983; Brown et al., 1984) and by ^{17}O labeling of phosphate groups for ^{31}P NMR studies of ActD–oligonucleotide complexes (Petersheim et al., 1984; Gorenstein et al., 1984). Most of these NMR studies were done with self-complementary oligomers which contained a signal GC binding site on the oligomer C_2 symmetry axis, and the results revealed that intercalation of the unsymmetrical phenoxazone ring destroyed the 2-fold symmetry of the duplexes.

Because of the complexity of the actinomycin molecule, it is not surprising that there is some scatter in the physical constants such as binding site size and specificity that characterize its interaction with DNA. Binding (Wells & Larson, 1970) and circular dichroism (Allen et al., 1976, 1977) studies with specific-sequence DNA polymers have clearly shown that ActD can bind to sequences other than GC. In addition, NMR studies on block copolymers (Early et al., 1977) and oligomers (Pardi et al., 1983) and DNase I footprinting experiments (Fox & Waring, 1984) have indicated that ActD binding to GC sites can cause structural, stability, and dynamic changes in surrounding sequences. The basic model emerging from these studies for ActD binding to DNA involves intercalation with high specificity at GC sites with approximately six base pairs being blocked by the cyclic peptides in the minor groove of the double helix (Waring, 1981). It should be noted, however, that Friedman and Manning (1984), on the basis of both an analysis of binding isotherms corrected for polyelectrolyte effects and model building studies, have proposed that ActD could bind at adjacent GCGC sites. It has been suggested that linking ActD and other base pair specific DNA binding agents together could create a set of sequence-specific DNA recognition molecules (Dervan, 1986). For rational design of the recognition molecules, it is essential to establish the binding site sizes of the individual units when they are present in high local relative concentrations (covalently linked).

We have undertaken a systematic NMR investigation of intercalator binding specificity and site size using a set of self-complementary oligomers containing both G·C and A·T binding regions (Figure 1). We have discovered that certain cationic porphyrin intercalators can exhibit very high specificity for CG sites and exclude binding at adjacent CG sites (Marzilli et al., 1986). The phenanthridinium intercalator propidium did not display any binding specificity with this same type of oligomer and bound by a simple neighbor exclusion mechanism (Wilson et al., 1986a). With the oligomer d(TGCGCA), we found that ActD not only intercalated at one GC site, as expected, but also could bind simultaneously at both of the GC sites (Wilson et al., 1986b). These preliminary results demonstrated that two different 1:1 ActD complexes were initially formed followed by a unique 2:1 complex at adjacent GC sites. These results have raised several important and interesting points concerning the ActD–DNA interaction which cannot be answered with self-complementary oligomers having a single 5′-GC-3′ site: (i) How does formation of the 1:1 and 2:1 complexes depend on oligomer length and sequence? (ii) Can more than two ActD bind at adjacent sites in a consecutive sequence of G·C base pairs? (iii) In a sequence of GC sites, are there binding preferences for the center or outer G·C sites? (iv) Are G·C and A·T base pairs adjacent to an ActD GC binding site perturbed in the same way by a bound ActD? These and other aspects of the ActD–DNA complexes have been investigated with the oligomers of Figure 1, and the results are reported here.

MATERIALS AND METHODS

Materials. The ^1H NMR spectrum of ActD (Sigma) agreed with literature results (Brown et al., 1984), and the compound was used without further purification. Stock solutions were made by dissolving ActD in water to a final concentration of approximately 1 mM. Final concentrations were calculated with an extinction coefficient of 24 500 M^{-1} cm^{-1} at 440 nm (Shafer et al., 1980). PIPES 10 buffer (10 mM PIPES, 1 mM EDTA, and 0.1 M NaCl, adjusted to pH 7.0 with NaOH) was used in all experiments. Oligonucleotides were synthesized and purified as previously described (Stec et al., 1985). NMR solutions were prepared by lyophilizing oligomer stock solutions, adding the appropriate amount of H_2O/D_2O and PIPES 10 to make a 0.6-mL sample approximately 20 mM in DNA bases, in PIPES 10 with 9% D_2O, and TSP as an internal reference. Oligonucleotide–ActD complex solutions were made by lyophilizing the appropriate amount of ActD from an H_2O solution and adding the oligonucleotide solution.

^1H NMR. Proton NMR spectra were obtained either at 270.15 MHz on a JEOL GX-270 or at 361.02 MHz on a Nicolet 360NB FT spectrometer (NMC 1280 software) by use of the Redfield 21412 pulse sequence to suppress the solvent signal as previously described (Wilson et al., 1986a). At 270.15 MHz, spectra were obtained under the following conditions: 5000 scans; 0.4-s pulse repetition; carrier frequency set at 13.4 ppm; 16K data points; TSP reference; 11 000-Hz spectral width; 0.6-mL sample volume in a 5-mm NMR tube. At 361.02 MHz typical experimental conditions were as follows: 2000 scans; 0.3-s pulse repetition; carrier frequency set at 14.4 ppm; 8K data points; TSP reference; 14 000-Hz spectral width; 0.6-mL sample volume in a 5-mm NMR tube. In the case of poly[d(G-C)]$_2$, three sets of 2000 transients were collected, block averaged in double precision mode, converted back to single precision, and Fourier transformed with 4-Hz line broadening. All imino protons were assigned by NOE and thermal denaturation techniques as previously described (Wilson et al., 1986a).

^{31}P NMR. Phosphorus NMR spectra were obtained at 81.01 MHz on an IBM WP-200SY spectrometer under the following experimental conditions: typically 10 000 scans; 45° flip angle; 2-s pulse repetition; broad-band proton decoupling; 2000-Hz spectral width; 1-Hz line broadening applied before Fourier transformation. Samples of 0.6 mL, contained in a 5-mm NMR tube, were inserted into a 10-mm tube containing PIPES 10/D_2O and trimethyl phosphate (TMP) as a reference. Some spectra were also determined at 101 MHz on a JEOL GX270 spectrometer under conditions similar to those described above.

Spectrophotometric Binding Isotherms. ActD binding isotherms with d(ATGCGCAT) were determined as previously described (Jones et al., 1986).

RESULTS

Oligomer Groups. There are two major groups of oligomers in Figure 1. The first group contains a central GCGC flanked by a variable-length sequence of A·T base pairs. This group was designed to evaluate length effects in formation of 1:1 and 2:1 ActD complexes at GCGC sequences, to determine how far away from the GC binding sites the conformational perturbations of ActD occur, and to establish a set of reference ^1H and ^{31}P spectra for the 1:1 and 2:1 complexes for use in analyzing results with other oligomers. NMR results with these oligomers were similar and will be presented only in general form below. There are two oligomers in the second group, one with three and one with four consecutive potential GC binding sites. These oligomers are directly related to d(TATGCGCATA) from group I and were designed to evaluate the long-range restrictions on ActD binding site size and binding cooperativity.

Length Effects in Formation of 1:1 and 2:1 Complexes. Comparison of the imino proton spectra for the group I oligomers (the first five oligomers of Figure 1) revealed some differences in the chemical shifts for the G·C signals. The pattern for the d(ATGCGCAT) oligomer, however, was also seen for the oligomers of 10 and 14 total base pairs which have additional flanking A·T base pairs, suggesting that end effects are not significant at low temperature for the central G·C base pairs when the oligomer length is eight base pairs or more. For this reason, the d(ATGCGCAT) oligomer was chosen for detailed imino proton spectral analysis of the effects of ActD on group I oligomers. Titration of d(ATGCGCAT) with ActD to the 1:1 ratio level (Figure 2) causes the appearance of three peaks in the A·T spectral region with seven resolvable peaks in the G·C shift range. There are two sets of peaks, one large and one small, as seen with d(TGCGCA) (Wilson et al., 1986b). The complexity of the spectrum indicates that the complex no longer has 2-fold symmetry and that the bound species are in slow exchange with free binding sites.

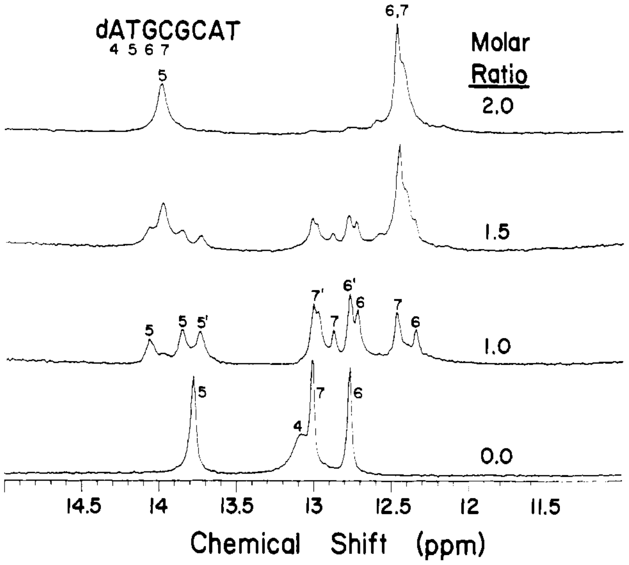


FIGURE 2: Imino proton spectra of ActD–d(ATGCGCAT)$_2$ complexes as a function of ratio. Assignments for the 0.0 and 2.0 ratio spectra are shown with the numbering scheme in the figure. The numbering scheme for the 1.0 ratio spectrum is given in the text. Because of the very large binding constant for ActD, the molar ratio is essentially the ratio of ActD bound per oligomer. It is possible that an additional 5′ peak (R = 1.0) is under the upfield 5 signal.

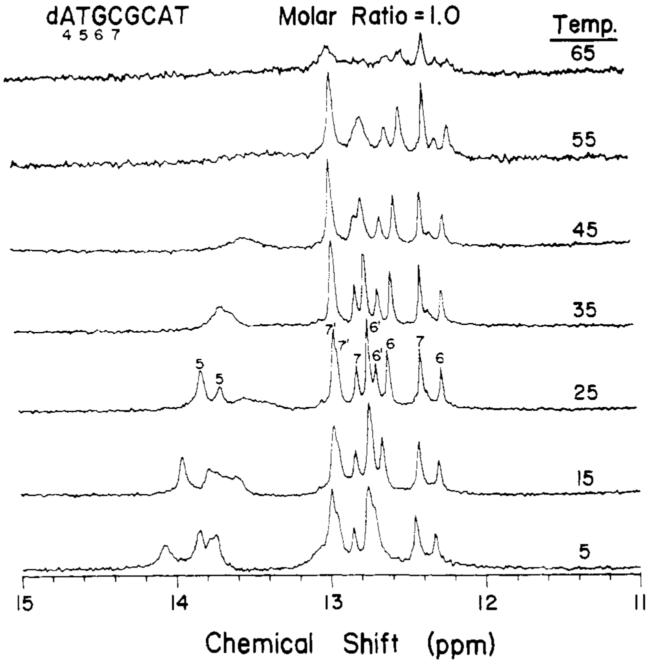


FIGURE 3: Imino proton spectra of ActD–d(ATGCGCAT)$_2$ at R = 1.0 as a function of temperature.

Spectra for the 1:1 ActD–d(ATGCGCAT) complex are shown from 5 to 65 °C in Figure 3. The increased resolution at higher temperature makes the spectral classification of signals of the adduct into two sets even more apparent. The benzenoid side of the phenoxazone ring of ActD (Figure 1) has a significantly larger ring current than the quinoid side (Giessner-Prettre & Pullman, 1976), and many of the observed shift differences in Figure 3 can be explained by the asym-

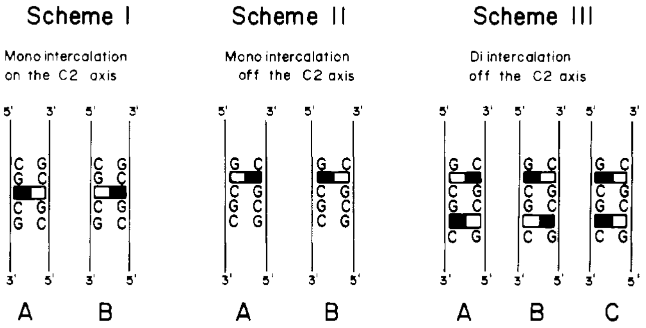


FIGURE 4: Schemes for the various intercalation modes of ActD with d(-CGCG-)- and d(-GCGC-)-type oligomers. The asymmetry of the ActD phenoxazone ring system is illustrated by dark- and light-shaded regions. Note that the two 1:1 complexes are equivalent with CGCG oligomers but are different with GCGC sequences.

metric shifts induced in the oligomer by the ActD molecule. The various possible orientations of the unsymmetrical phenoxazone ring in binding sites on and off of an oligomer C_2 axis are shown in Figure 4. The pairs of peaks with different intensities in both ^1H and ^{31}P (see below) spectra for 1:1 complexes (note, for example, the 25 °C spectrum in Figure 3) suggest that the two 1:1 complexes are not present in equal amounts.

The temperature-dependent spectra in Figure 3 can be interpreted with the aid of the scheme

4 5 6 7 7′ 6′ 5′ 4′
d A T G|C G C A T

which uses the numbering convention of Figure 1 and has ActD (vertical line) bound at only one GC site. The spectra could have up to 16 imino proton signals due to the two possible orientations of bound ActD. Broad signals for the terminal, 4 and 4′, A·T base pairs can be seen slightly above 13 ppm in the 5 °C spectrum in Figure 3, but these are totally broadened by 15 °C. The 5′ A·T base pair is not significantly shifted on addition of ActD and shows the next higher T_m. In the 25 °C spectrum the two different families of signal intensities for the two different orientations of ActD in 1:1 complexes can be easily seen. Compare, for example, the two most downfield A·T signals with the two most upfield G·C signals in that spectrum.

At 25 °C three base pair imino proton signals are lost due to thermally induced end fraying, and five are still in slow exchange with water. Due to the two orientations of the ActD ring, these five base pairs give 10 resolved signals at 25 °C. The two most downfield peaks, for A·T base pair 5, cannot be shifted due to ring current effects of the intercalated ActD, which would cause upfield shifts of these imino protons (Krugh & Nuss, 1979; Patel, 1974; Wilson et al., 1986a). These downfield shifts suggest that ActD causes significant changes in the oligomer base stacking and/or H bonding of the flanking region. These changes are also different for the two orientations of the bound ActD molecule.

The eight peaks in the upfield region of the spectrum at 25 °C are due to G·C base pairs 6, 7, 7′, and 6′. Smaller shifts are predicted for the 7′- and 6′-protons than for the 6- and 7-protons at the ActD binding site. The large peak at approximately 13 ppm at 5 °C contains 7′-protons for both 1:1 complexes. These two are partially resolved at 15 and 25 °C. In the same manner the large peak at approximately 12.7 ppm contains signals for both 6′-protons, and these are well-resolved at 25 and 35 °C. Irradiation of the 7′-protons gives an NOE to the 6′-protons and a proton at approximately 12.4 ppm at 5 °C, which must be one of the 7-protons. Because of the larger ring current effect of the benzenoid portion of the phenoxazone ring of ActD (Giessner-Prettre & Pullman, 1976), this 7-proton is tentatively assigned to the 1:1 complex with the benzenoid portion of the ActD ring over the G in G·C base pair 7. The most upfield proton is, thus, assigned to G·C-6 in the 1:1 complex with the benzenoid part of the ActD ring over the G in G·C-6. There must be a lower intensity G-7 proton and a higher intensity G-6 proton characteristic of the smaller shifts due to the quinoid portion of the phenoxazone ring system in the two complexes. These are clearly resolved in the 25 °C spectrum in Figure 3 and are labeled.

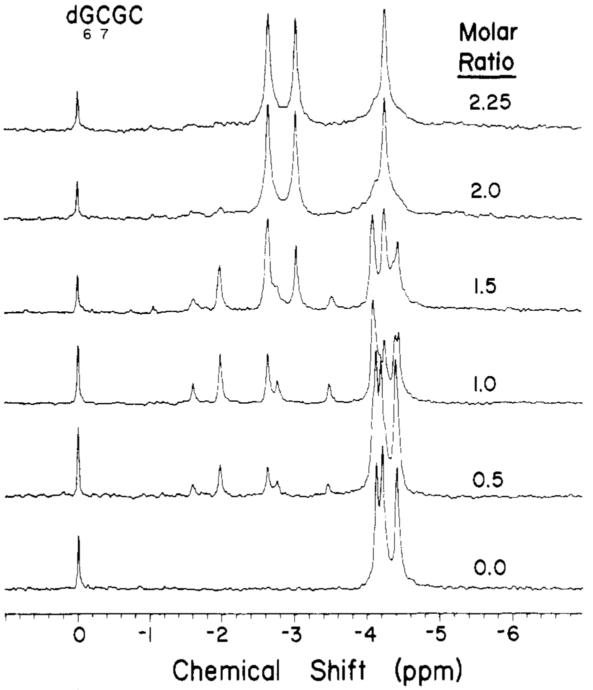


FIGURE 5: ^{31}P spectra of ActD–d(GCGC)$_2$ complexes as a function of ratio.

At $R = 1.5$ and 2.0, two new signals are observed, one at 13.94 ppm in the A·T region and one at 12.42 ppm in the G·C region, and the signals seen in the $R = 1.0$ spectrum lose intensity (Figure 2). The peak at 13.93 ppm is assigned to A·T-5, and overlapping peaks near 12.4 ppm are assigned to signals for G·C base pairs 6 and 7. The return to a less complex spectrum is seen for all five oligomers with the central GCGC sequence and indicates that C_2 symmetry is regained in the 2:1 complex. The larger upfield shift for G in G·C-7 relative to G·C-6 in the 2:1 complex suggests, but does not prove, that the benzenoid side of the ActD ring system is over G in G·C-7 as in the most stable 1:1 complex.

Oligomers with additional A·T base pairs (Figure 1) gave results similar to those obtained with d(ATGCGCAT). The long-range effects of binding ActD cause upfield shifts of A·T-4 in both the 1:1 and 2:1 complexes in contrast to the downfield shifts for A·T-5, and the effects at A·T-4 are more easily seen in the longer oligomers. ActD obviously has significant effects on the shifts of base pairs away from the binding site, and these long-range as well as local steric effects, no doubt, account for the formation of the unique 2:1 complex with all five of the oligomers of this group.

The oligomer d(GCGC) has three phosphates, and signals for these are resolved in the ^{31}P NMR spectrum (Figure 5, bottom spectrum) at −4.13, −4.20, and −4.40 ppm. In the titration with ActD, the spectra at $R = 0.5$ and 1.0 (Figure 5) have five new downfield signals which are quite similar to those previously assigned for d(TGCGCA) (Wilson et al., 1986b). Changes in the original spectral region (−4.1 to −4.5

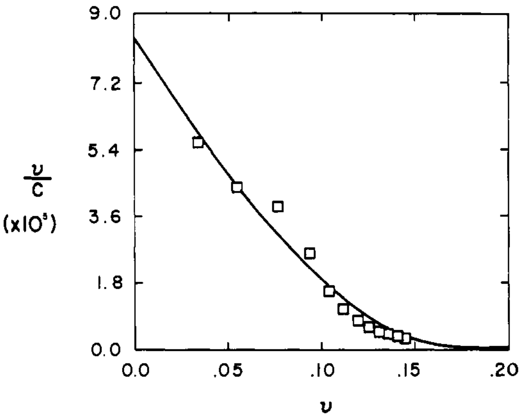


FIGURE 6: Scatchard plot for binding of ActD to d(ATGCGCAT) at 15 °C in PIPES 10 buffer. The points in the figure are experimental, and the line is the nonlinear least-squares best fit value with the cooperative model of McGhee and von Hippel (1974). The fitting is only approximate since an oligomer with only two sites instead of a polymer is being used.

ppm) indicate that there are also new signals in this region. In agreement with the imino proton results, the downfield peaks consist of a set of large peaks and a set of smaller peaks which are characteristic of the two different ActD 1:1 complexes. At $R > 1.0$, two additional new downfield peaks emerge, again as previously observed with d(TGCGCA) (Wilson et al., 1986b), and the spectrum at $R = 2.0$ has three major resonances at −4.26, −3.03, and −2.65 ppm. There is still some hint of the signals that emerged at $R = 0.5$–1.0. At $R = 2.25$, only the three major peaks observed at $R = 2.0$ remain, indicating that C_2 symmetry has been restored by the formation of a unique 2:1 species as suggested by the imino proton results. In analogy with d(TGCGCA), the upfield phosphate signal is tentatively assigned to P-2 (G-1-C-2-G-3-C) while the most downfield signal is for P-1, the 5′-phosphodiester, and the central signal is for the 3′-phosphodiester, P-3. The appearance of signals for bound and free states in the ^{31}P spectra indicates, as with the ^{1}H results, that complexed and free sites are in slow exchange. ^{31}P spectra for the longer oligomer–ActD complexes are very similar in the downfield region.

In order to obtain an estimate for the relative binding constants for ActD in the 1:1 and 2:1 complexes, we conducted spectrophotometric binding experiments at 15 °C in PIPES 10 buffer with the d(ATGCGCAT) oligomer. We made the usual assumption that the bound extinction coefficient of ActD is the same in all complexes. Since ActD binds in the center of the oligomer, end effects should not have a large effect on the equilibria. At NMR concentrations, greater than 99% of ActD is bound, and no accurate binding isotherm can be obtained due to the large errors introduced by small errors in the bound extinction coefficient under these conditions (Deranleau, 1969; Wilson & Lopp, 1979). Very reproducible binding isotherms could be obtained at lower concentration, and an example Scatchard plot is shown in Figure 6. These results were fitted with a nonlinear least-squares program using the cooperative, site-exclusion model of McGhee and von Hippel (1974). The apparent binding constant obtained in this manner is 8.4×10^5 M^{-1}, the number of base pairs per binding site is 4.2, in good agreement with the expected value of 4 [2 ActD/8 base pairs in d(ATGCGCAT)], and the cooperativity parameter is 0.047, indicating pronounced anticooperativity in binding of the second ActD. The cooperativity parameter indicates that the net binding constant for ActD entering the second GC site in d(ATGCGCAT) is only approximately $^1/_{20}$ the value for binding to the first site. Since the two 1:1 complexes have similar NMR signal intensities with this oligomer, no correction was made for their binding differences or for the two orientations of ActD to give the two 1:1 complexes. These subtle effects are not resolved in the spectrophotometric analysis. It is impossible to predict the formation of the two asymmetric 1:1 and unique 2:1 complexes from the Scatchard plot, but these results are clear from the NMR experiments. The signal intensity differences in NMR spectra of the 1:1 complexes suggest that these equilibrium constants differ by only a factor of 2–3.

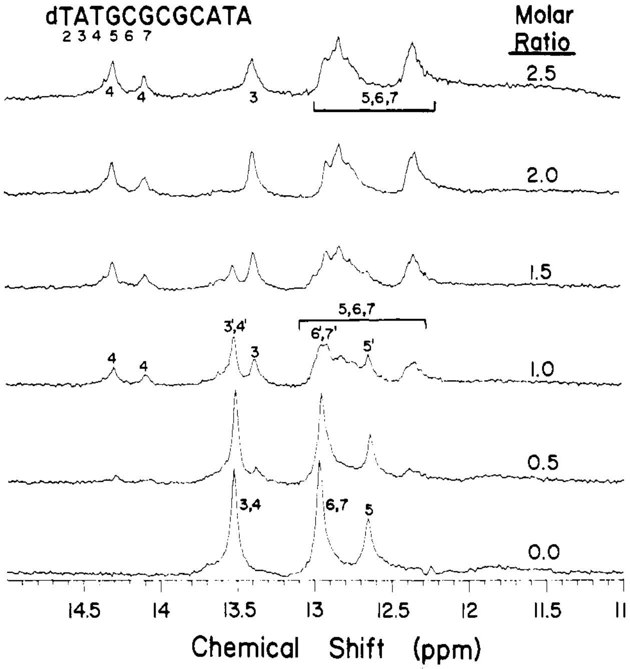


FIGURE 7: Imino proton spectra of ActD–d(TATGCGCGCATA)$_2$ complexes as a function of ratio. Assignments for the free oligomer and 2:1 complexes are shown with the numbering scheme in the figure. The numbering scheme for the $R = 1.0$ spectrum is given in the text. Specific assignments cannot be made for the 5-, 6-, and 7-protons due to extensive peak overlap.

Effect of Increasing Numbers of Adjacent GC Sites. Three oligomers in Figure 1 have the general structure d[TAT(GC)$_n$ATA], where $n = 2$, 3, and 4. ActD binds strongly to isolated GC sites, and we have convincingly demonstrated above that ActD can form a 2:1 complex with the $n = 2$ and other oligomers of group I. Binding of the second ActD to the $n = 2$ oligomer is, however, highly anticooperative, and the $n = 3$ and 4 oligomers have been synthesized to determine what restrictions there are on the long-range ability of ActD to bind at adjacent GC sites.

The imino region of the proton spectrum of d(TATGCGCGCATA) (Figure 7, bottom spectrum) has three resolved peaks. The signal at 13.5 ppm at 5 °C is assigned to the coincident peaks belonging to the two types of internal A·T base pairs. A very broad signal for the terminal A·T base pair is between 11.5 and 12.0 ppm. The signal for A·T base pair 3 (Figure 1) broadens and shifts upfield at 20–30 °C, and the signal for base pair 4 does the same in the 30–40 °C range (not shown). In the G·C region, the small peak at 12.65 ppm has been assinged to the G·C base pair adjacent to the A·T base pair, on the basis of chemical shift, thermal melting, and analogy with results for d(TATGCGCATA). The large peak at 12.96 ppm integrates as two protons and is assigned to the two types of internal G·C base pairs. These remain as a single peak to >50 °C.

Both upfield and downfield shifts are seen (Figure 7) on addition of ActD to d(TATGCGCGCATA). On the basis

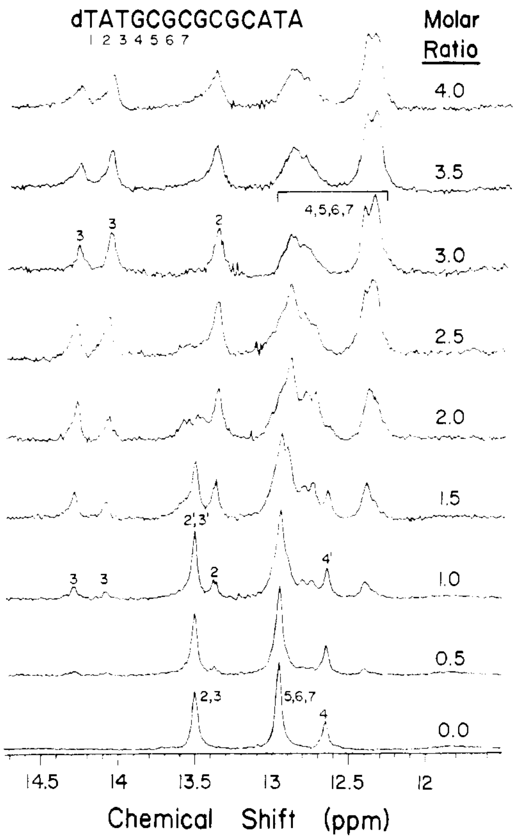


FIGURE 8: Imino proton spectra of ActD–d(TATGCGCGCGCATA)$_2$ complexes as a function of ratio. Protons for the free oligomer are assigned with the numbering scheme in the figure. In the spectrum at a molar ratio of 1.0, protons labeled 2 and 3 are for A·T base pairs next to an ActD bound at the 4–5 GC site. Protons labeled 2′, 3′, and 4′ are at the other end of the oligomer and have shifts very similar to the original values. In the 3.0 molar ratio spectrum, assignments are shown with the numbering scheme in the figure. Specific assignments for protons 4, 5, 6, and 7 cannot be made.

of the results discussed above, the two most downfield peaks are characteristic of the proton for A·T base pair 4 with ActD bound in two 1:1 complexes at the 5–6 GC site:

2 3 4 5 6 7 7′ 6′ 5′ 4′ 3′ 2′
d T A T G | C G C G C A T A

where the vertical line indicates a bound ActD. Peak assignments are shown in Figure 7 for reference. The decrease in intensity of the signals for the free A·T-3 and -4 and G·C-5 base pairs can be clearly seen. The new signal at approximately 13.4 ppm is assigned to A·T base pair 3 near an ActD–GC 5–6 binding site. The most upfield peaks are characteristic of G·C protons at ActD binding sites. Increasing the ratio to 2.0 leads to essentially complete disappearance of the signals for the free A·T-3 and A·T-4 and G·C-5 base pairs, an increase in intensity of the upfield and downfield signals that appeared in the 1.0 ratio spectrum, and little other difference between the 1.0 and 2.0 ratio spectra. The complete disappearance of the signals for the free A·T-3 and -4 base pairs indicates that both ActD are bound at the outer GC binding sites and that essentially no ActD is bound at the central GC site. This is expected from the negative cooperativity observed for binding of the second ActD in the oligomers with only two adjacent GC sites. Addition of ActD above the 2.0 ratio did not result in any significant shifts relative to the 2.0 ratio spectrum. Above a ratio of 2.5, the spectra became broader, suggesting some aggregation, but this broadening could be reversed by increasing the temperature. Thus, ActD preferentially binds at both outer GC sites, each giving two 1:1 (isolated) type complexes. Up to essentially the solubility limit of ActD, however, this oligomer will not form a 3:1 complex by addition of an ActD to the center GC site.

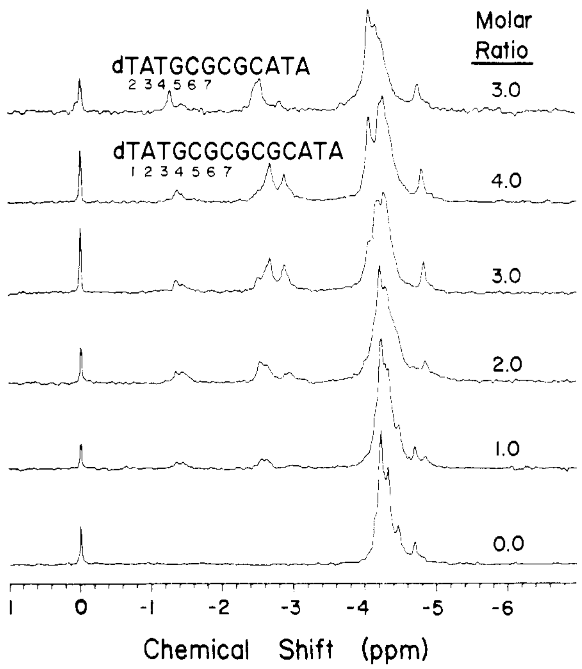


FIGURE 9: The bottom five ^{31}P spectra are of the ActD–d(TATGCGCGCGCATA)$_2$ complexes as a function of ratio. The top spectrum is of the ActD–d(TATGCGCGCATA)$_2$ complex at R = 3.0.

The imino proton spectrum for d(TATGCGCGCGCATA) (Figure 8) is essentially identical with that for d(TATGCGCGCATA) (Figure 7) except that the G·C signal intensity at 12.96 ppm increases in intensity due to the addition of another internal G·C base pair. The integrated intensity ratio for the signals at 12.96–12.65 ppm is 3:1 as expected for the three internal to one external G·C base pair ratio in the oligomer. The signals for the two internal A·T base pairs (2 and 3) overlap at 13.5 ppm as with d(TATGCGCGCATA), and the terminal base pair signal is a broad, barely visible, peak at 11.5–12.0 ppm. The similarity of chemical shifts and intensity changes observed with this oligomer supports the assignments discussed above for d(TATGCGCGCATA).

The addition of ActD to d(TATGCGCGCGCATA) to ratios of 0.5–1.0 (Figure 8) results in new peaks below 14.0 ppm, as well as upfield peaks around 12.4 ppm. At R = 1.5 and 2.0 all the new peaks increase and the parent signals decrease in intensity. Between ratio 2.0 and ratio 3.0, the two most downfield peaks switch intensity ratios with the peak at 14.1 ppm becoming larger than the peak at 14.3 ppm. At R = 3.0, the original signals for A·T base pairs 2 and 3 are gone, and there is a very broad resonance, made up of several peaks, in the G·C region from 12.7 to 13.0 ppm. The spectra from R = 3.0 to R = 5.0 basically do not change except for broadening at the higher ratios. Heating the R = 5.0 sample results in initial upfield shifts and broadening of the peaks near 13.5 ppm (not shown). This is followed, with increasing temperature, by upfield shifts and broadening of the signals at 14 ppm as expected from their assignment to A·T base pairs. Some slight sharpening of the G·C signals is observed in this same temperature range.

Thirteen phosphorus peaks are expected in the ^{31}P spectrum of d(TATGCGCGCGCATA) and appear in an overlapping band in the −4.2 to −4.7 ppm region (Figure 9, bottom spec-

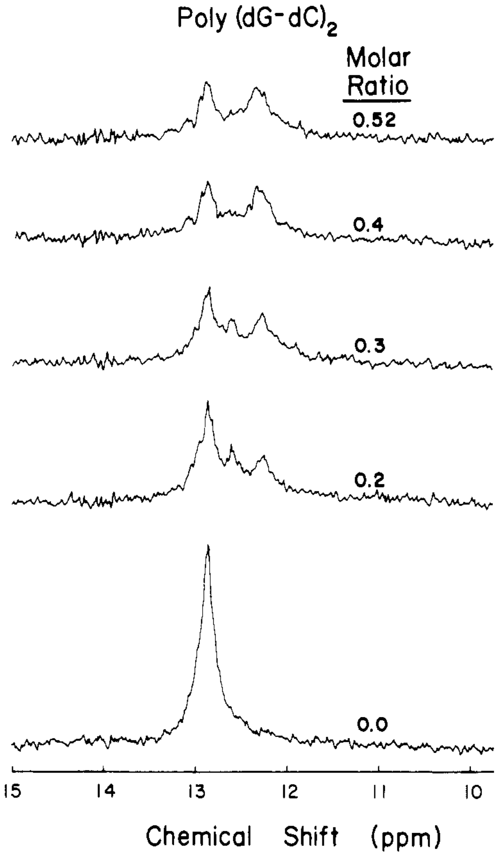


FIGURE 10: Imino proton spectra of ActD–poly[d(GC)]$_2$ as a function of ratio at 50 °C. The concentration of the polymer was 4.7 mM in DNA phosphates, and each spectrum is for 6000 scans. Little change is seen in the spectra above the 0.4 ratio, suggesting that saturation of the polymer occurs between the 0.3 and 0.4 ratio.

trum). The addition of ActD to $R = 2.0$ results in the emergence of a new upfield peak at −4.82 ppm and several downfield signals. At $R = 3.0$, new signals at −2.65 and −2.80 ppm are predominant while the other downfield peaks have lost intensity. At $R > 3$, the two peaks at −2.65 and −2.80 ppm remain predominant and the small peaks remain largely unchanged. The two signals at −2.65 and −2.80 ppm are indicative of a 2:1 (adjacent) type species in the GCGC series oligomers as discussed above. The downfield signals near −1.3 ppm are an indication of the presence of one or more 1:1 (adjacent) type species. These results and the constancy of the spectra above a 3:1 ratio suggest that the final complex of this oligomer with ActD contains three ActD molecules in a combined 2:1–1:1 (adjacent–isolated) type complex:

d(TATG|CGCG|CG|CATA)

where the lines indicate bound ActD. The spectrum of the d(TATGCGCGCATA) oligomer with $R = 3$ is shown at the top of Figure 9, and as indicated by imino proton results, appears to be composed of two isolated-type complexes.

Multiple Adjacent GC Sites: *Poly*[*d*(*G-C*)]$_2$. The low-field region of the proton spectrum is shown in Figure 10 for the titration of sonicated poly[d(G-C)]$_2$ with ActD. The spectrum for the uncomplexed polymer has a single peak at 12.92 ppm. At intermediate ratios the original peak is reduced in intensity, and two new peaks are seen at 12.76 and 12.42 ppm. For the neighbor exclusion model with simple intercalators, the saturation ratio is predicted to be 0.5 (intercalator per base pair). As this ratio is approached, the peak at 12.76 is reduced in intensity. The spectrum at the ratio of 0.54 has two peaks of approximately equal intensity at 12.87 and 12.33 ppm. The oligomer of Figure 1 which is most similar to the polymer is d(TATGCGCGCGCATA), and it is interesting to compare the oligomer and polymer titration results with ActD. Signals for internal G·C base pairs 5, 6, and 7 in the free oligomer are near 13 ppm as is the polymer imino proton signal. Signals for base pairs 2, 3, and 4 in the oligomer are not relevant since they are for A·T base pairs or a G·C base pair next to A·T base pairs. On addition of ActD, the G·C base pairs of the oligomer give complex upfield shifts as do those of the polymer. At high ratios of ActD, the G·C base pairs of both the oligomer and polymer give broad signals between 12 and 13 ppm. We have suggested that the final complex with the oligomer is a mixture of isolated- and adjacent-type complexes, and the similar G·C spectral results also suggest that the polymer complex is a similar mixture.

At 30 °C the ^{31}P NMR spectrum of poly[d(G-C)]$_2$ has a single broad peak at −4.22 ppm (not shown). At a ratio of 0.20 ActD per base pair, the upfield peak at −4.22 is reduced in intensity, and a new, small broad peak appears at −2.8 ppm. At the final ratio of 0.54 ActD per base pair, the upfield resonance is shifted to −4.10 ppm and the broad downfield peak is shifted to −2.7 ppm. Upon heating this sample to 50 °C, both peaks sharpened with the downfield peak shifting to −2.5 ppm. At high ratios of ActD, d(TATGCGCGCGCATA) has its major downfield ^{31}P intensity in bands between −2.5 and −3.0 ppm (Figure 9).

DISCUSSION

NMR (Krugh & Nuss, 1979; Patel, 1974; Patel et al., 1981; Reid et al., 1983; Brown et al., 1984; Petersheim et al., 1984; Gorenstein et al., 1984), crystallographic (Sobell, 1973; Takusagawa & Berman, 1983), and theoretical (Lybrand et al., 1986) studies have indicated that ActD binds preferentially to GpC sites in DNA. These studies have suggested that this preference is due to formation of ActD-G hydrogen bonds: a proton of the 2-amino group of G with the carbonyl oxygen of threonine; the N-3 ring nitrogen of G with a threonine amide hydrogen in the cyclic peptide group of ActD. This model has been generally supported by binding (Wells & Larson, 1970), footprinting (Fox & Waring, 1984), and other spectroscopic (Allen et al., 1976, 1977) analyses and has led to the consensus that ActD binds preferentially to GC sites and blocks a total of approximately six base pairs in its DNA complex [reviewed in Waring (1981)]. Friedman and Manning (1984), however, have proposed that ActD requires as few as two base pairs for a binding site. The NMR studies reported here are the first with oligomers containing multiple GC sites. These studies provide considerable new information on the multitude of possible ActD–DNA complexes and indicate why some disagreement has arisen over the years in the determination of physical characteristics of the ActD–DNA complex, such as the number of base pairs blocked by a bound ActD.

Although the two cyclic peptide rings of ActD are the same, the phenoxazone ring is not symmetrical (Figure 1), and ActD can have two orientations when intercalated into a GC site (Figure 4). If the GC site is on an oligomer C_2 symmetry axis, the two orientations will be equivalent (Figure 4) as in most oligomer NMR studies to this time. The GC binding sites in the GCGC oligomers of Figure 1 are off the C_2 axis, and the two orientations of ActD can be easily detected. In the NMR spectra for 1:1 complexes, we obtained two sets of peaks, which did not have the same area. This indicates that both possible complexes are formed and that they do not have the same free energy for formation although the difference is quite small. This is the first direct observation of the two possible orientations of an asymmetric intercalator. Chemical shift and intensity arguments, based on the asymmetric shifts induced by the phenoxazone, suggest that the orientation of ActD in the 1:1 complexes with the benzenoid ring of the phenoxazone system over the G in GC base pair 7 in the oligomers of Figure 1 is preferred by a factor of 2–3 to 1.

Several important new features of the ActD–DNA interaction emerge from the results with the 1:1 complexes. First, with ActD bound at a site of the type

4 5 6 7 7′ 6′ 5′ 4′
...A T G | C G C A T...

where the vertical line indicates ActD, the shifts for A·T base pair 5 are downfield while the shifts for A·T-4 and G·C-7′ are upfield. With oligomers containing central CGCG sequences (instead of GCGC) with flanking A·T base pairs, ActD binding at the GC site induces only upfield shifts in flanking G·C and A·T base pairs (Wilson et al., 1986b; Jones, Scott, Zon, Marzilli, and Wilson, unpublished results). This indicates that the effects of bound ActD on adjacent base pairs will strongly depend on the surrounding oligomer sequence. Second, the induced shifts for the two orientations of bound ActD in 1:1 complexes are significantly different. This difference can be seen most easily by looking at the shift difference in the signals for A·T base pair 5 (see Figure 2 for example) or in the phosphorus spectra for any 1:1 complex. In X-ray structures of ActD stacked with G (Sobell, 1973) or G·C base pairs (Takusagawa & Berman, 1983), the quinoid portion of the phenoxazone ring system is significantly twisted with respect to the planar benzenoid portion of the ring. Since this twisting of the ring system occurs in stacked complexes, it is reasonable that it also occurs at intercalation sites. This structural asymmetry as well as differences in ring current effects may account for the different shifts induced in ^{1}H and ^{31}P spectra by ActD bound in the two different 1:1 complexes.

The downfield region of the 1:1 ^{31}P NMR spectra for ActD bound to all of the GCGC oligomers is quite similar and contains more signals than for the phosphodiesters at the intercalation site. With d(TGCGCA) the phosphate signals have been assigned with ^{17}O labeling (Wilson et al., 1986b). The most downfield signals are for the phosphate groups at the intercalation site, and the additional downfield peaks are found to be due to phosphodiesters adjacent to the intercalated ActD. Patel (1986) also saw an additional downfield peak in the ^{31}P spectrum of ActD complexed to d(ATGCAT) and suggested that the extra peak was due to nonintercalated ActD. On the basis of our results with 1:1 complexes, however, we suggest that extra downfield peaks are a characteristic of ActD–DNA 1:1 complexes and arise from long-range structural perturbations of the DNA backbone by bound ActD. It is interesting that only two downfield peaks are seen in the 2:1 spectra and that these two peaks, for the two phosphodiesters at the intercalation sites (Wilson et al., 1986b), are closer together than the peaks for the same two phosphate groups in the 1:1 complexes. This diversity of shifts for the phosphates at the GC intercalation site indicates that the shift differences cannot be solely due to the asymmetric ring current across the phenoxazone ring of ActD and that the two 1:1 and the 2:1 complexes must have different intercalation conformations.

Perturbations of neighboring base pairs are also observed in the 2:1 ActD complexes. Binding of two ActD at the GC 6–7 site in d(TATGCGCATA) causes large downfield shifts in the signal of A·T base pair 5 and smaller upfield shifts in the signal for A·T base pair 4. In the 2:1 complex of ActD at the two GC sites 5–6 in the oligomer d(TATGCGCGCATA) (Figure 7), downfield shifts of A·T base pair 4 are seen as expected, but there is no downfield shift of G·C base pair 7 on the opposite side of the intercalation complex. This again illustrates that A·T and G·C pairs neighboring an ActD binding site are affected differently when ActD is bound. The upfield shift of A·T base pair 3 in this complex also illustrates that the long-range effects of a bound ActD depend on the surrounding sequence.

Formation of the unique 2:1 complex is anticooperative as can be seen most easily with the titration results for the simple d(GCGC) oligomer in Figure 5. At the 0.5 ratio, free duplex and 1:1 adduct, but no 2:1 adduct, signals are seen. At the 1.0 ratio, the free signals have disappeared, indicating perturbation of all base pairs by a single bound ActD, and 1:1 but no 2:1 complex signals can be detected within the error limits of the NMR method. Increasing the ratio of ActD leads to stoichiometric formation of the 2:1 complex. Similar results were obtained with all members of the first group of oligomers of Figure 1. Spectrophotometric binding studies (Figure 6) also indicate that binding of the second ActD in GCGC sites is highly anticooperative. This negative cooperativity in binding of the second ActD is probably partially due to steric interference of the first bound ActD and to the configurational entropy loss due to formation of a unique 2:1 complex.

Characteristic ^{31}P and ^{1}H spectra obtained for the 1:1 (isolated) and 2:1 (adjacent) type complexes with the first group of oligomers can be used in analyzing the spectra for ActD complexed with the second group of oligomers containing the longer GC segments. With the oligomer d(TATGCGCGCATA) only 1:1 (isolated) complex type spectra are obtained even on addition of an excess of ActD. Saturation binding is obtained at two ActD per duplex. Chemical shift changes in the A·T region indicate that ActD can bind at both outer GC sites, but when these are filled, no significant binding at the center GC occurs. This is an extreme case of anticooperativity, presumably because additional ActD binding in the accessible concentration range is prohibited by the double steric hindrance of the two bound ActD. With the oligomer d(TATGCGCGCGCATA), mixed isolated and adjacent complex type spectra are obtained, and binding saturates at three ActD per duplex. Chemical shifts in the A·T region indicate that, at three ActD bound per duplex, two ActD are bound to the outer GC sites. One ActD can then bind to either of the two center GC sites. The next ActD would then have to bind between two bound ActD, but as with the oligomer with three GC sites, this does not occur. There is essentially total anticooperativity to binding of ActD between two molecules of ActD already bound adjacent to the potential binding site. An ActD can, thus, have one bound neighbor but not two. Information of this type is essential in the design of complex sequence recognition molecules containing more than one derivative of ActD. Results obtained with the ActD complex of poly[d(G-C)]$_2$ are quite similar to those with the four GC site oligomer.

In conclusion, we strongly support the GC binding preference of ActD irrespective of sequence length and of surrounding sequence (of those investigated). Sequences such as CG and AT/TA type sites were much less preferred than the GC binding site in the oligomers of Figure 1 and others we have investigated (Wilson et al., 1986b). At the GC site ActD can bind in two possible orientations (Figure 4) with the peptides in the minor groove. We have observed both complexes, and they have binding equilibrium constants that differ by only a factor of 2 or 3. Friedman and Manning (1984) predicted that ActD could bind at adjacent GC sites on DNA but with some distortion of the ActD peptides. Our

NMR results clearly demonstrate that ActD can bind at adjacent sites but only in one orientation and with some distortion of both the adjacent and neighboring base pairs. In longer GC sequences only two ActD can bind to every three GC sites. Both the favored 1:1 and the unique 2:1 complex in the oligomer with a central GCGC have the benzenoid side of the phenoxazone ring over the G in base pair 7 (Figure 1). The reasons for the enhanced stability of ActD oriented in this way are not clear at present but are being investigated by 2D NMR methods.

REFERENCES

Allen, F. S., Moen, R. P., & Hollstein, U. (1976) *J. Am. Chem. Soc. 98*, 864–865.

Allen, F. S., Jones, M. B., & Hollstein, U. (1977) *Biophys. J. 20*, 69–78.

Brown, S. C., Mullis, K., Levenson, C., & Shafer, R. H. (1984) *Biochemistry 23*, 403–408.

Deranleau, D. (1969) *J. Am. Chem. Soc. 91*, 4044–4049.

Dervan, P. B. (1986) *Science (Washington, D.C.) 232*, 464–471.

Early, E. A., Kearns, D. R., Burd, J. F., Larson, J. E., & Wells, R. D. (1977) *Biochemistry 16*, 541–551.

Fox, K. R., & Waring, M. J. (1984) *Nucleic Acids Res. 12*, 9271–9285.

Friedman, R. A. G., & Manning, G. S. (1984) *Biopolymers 23*, 2671–2714.

Giessner-Prettre, C., & Pullman, B. (1976) *C. R. Seances Acad. Sci., Ser. D 283*, 675–676.

Gorenstein, D. G., Lai, K., & Shah, D. O. (1984) *Biochemistry 23*, 6717–6723.

Jones, R. L., Zon, G., Krishnamoorthy, C. R., & Wilson, W. D. (1986) *Biochemistry 25*, 7431–7439.

Krugh, T. R., & Nuss, M. E. (1979) in *Biological Applications of Magnetic Resonance* (Schulman, R. G., Ed.) Chapter 3, Academic, New York.

Lybrand, T. P., Brown, S. C., Creighton, S., Shafer, R. H., & Kollman, P. A. (1986) *J. Mol. Biol. 191*, 495–507.

Marzilli, L. G., Banville, D. L., Zon, G., & Wilson, W. D. (1986) *J. Am. Chem. Soc. 108*, 4188–4192.

McGhee, J. D., & von Hippel, P. H. (1974) *J. Mol. Biol. 86*, 469–489.

Müller, W., & Crothers, D. M. (1968) *J. Mol. Biol. 35*, 251–290.

Pardi, A., Morden, K. M., Patel, D. J., & Tinoco, I. (1983) *Biochemistry 22*, 1107–1113.

Patel, D. J. (1974) *Biochemistry 13*, 2396–2402.

Patel, D. J., Koglowski, S. A., Rice, J. A., Broka, C., & Itakura, K. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 7281–7284.

Petersheim, M., Mehdi, S., & Gerlt, J. A. (1984) *J. Am. Chem. Soc. 106*, 439–440.

Reid, D. G., Salisburg, S. A., & Williams, D. H. (1983) *Biochemistry 22*, 1377–1385.

Shafer, R. H., Burnette, R. R., & Mirau, P. A. (1980) *Nucleic Acids Res. 8*, 1121–1131.

Sobell, H. M. (1973) *Prog. Nucleic Acid Res. Mol. Biol. 13*, 153–190.

Stec, W. J., Zon, G., Egan, W., Byrd, R. A., Phillips, L. R., & Gallo, K. A. (1985) *J. Org. Chem. 50*, 3908–3913.

Takusagawa, F., & Berman, H. (1983) *Cold Spring Harbor Symp. Quant. Biol. 47*, 315–321.

Waring, M. (1970) *J. Mol. Biol. 54*, 247–279.

Waring, M. (1981) in *The Molecular Basis of Antibiotic Action* (Gale, F., et al., Eds.) pp 314–333, Wiley, London.

Wells, R. D., & Larson, J. E. (1970) *J. Mol. Biol. 49*, 319–342.

Wilson, W. D., & Lopp, I. G. (1979) *Biopolymers 18*, 3025–3041.

Wilson, W. D., Jones, R. L., Zon, G., Banville, D. L., & Marzilli, L. G. (1986a) *Biopolymers 25*, 1997–2015.

Wilson, W. D., Jones, R. L., Zon, G., Scott, E. V., Banville, D. L., & Marzilli, L. G. (1986b) *J. Am. Chem. Soc. 108*, 7113–7119.