Actinomycin D–Deoxydinucleotide Interactions as a Model for Binding of the Drug to Deoxyribonucleic Acid. Proton Magnetic Resonance Results†

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ABSTRACT: The interactions of actinomycin D and a series of eight deoxydinucleotides have been studied by 100-MHz proton magnetic resonance. The spectra were recorded as a function of the nucleotide to drug ratio to determine the stoichiometry of complex formation. The results show that actinomycin D has two binding sites for guanine-containing nucleotides. The deoxydinucleotide pdG-dC forms an intercalated-type complex with actinomycin D. The geometries of the complexes were deduced from an analysis of the induced shifts of the actinomycin D protons upon complex formation with the dinucleotides. A model for the interaction of the deoxydinucleotides with actinomycin D is proposed. This model is consistent with both the present nuclear magnetic resonance experiments and the results from the visible spectra (Krugh, T. R. (1972), *Proc. Nat. Acad. Sci. U. S. 69*, 1911). The present results also illustrate the general utility of the deoxydinucleotides as models for studying drug–DNA interactions.

Actinomycin D (Figure 1) is an antibiotic that binds to DNA and specifically inhibits RNA synthesis (Kirk, 1960; Kersten *et al.*, 1960; Reich *et al.*, 1961). The interaction of actinomycin D and DNA has been widely studied by a number of techniques (for reviews, see Reich and Goldberg, 1964, Goldberg and Friedman, 1971, and Sobell, 1973). The change in the visible spectrum of actinomycin D as it binds to DNA provides a convenient means of monitoring complex formation. These changes may be mimicked by complexing actinomycin D with a variety of mononucleosides or nucleotides (Kersten, 1961; Reich, 1964; Reich and Goldberg, 1964) but the apparent binding constants are approximately three orders of magnitude smaller than the binding constants for the interaction of actinomycin D with DNA. We have recently reported the results of an investigation of the interaction of actinomycin D and a series of deoxydinucleotides (Krugh, 1972) using optical spectral techniques. We found that actinomycin D has a strong preference for complexing with a GpC sequence. This preference for the GpC sequence was proposed by Sobell and Jain (1972) in their model for the interaction of actinomycin D and DNA. Their model proposed that the phenoxazone ring of actinomycin D intercalates into the DNA helix with the cyclic pentapeptide rings located in the minor groove of the DNA helix. The intercalation model had been proposed earlier by Müller and Crothers (1968) on the basis of kinetic and hydrodynamic studies. Support for the intercalation model has come from the binding studies of actinomycin to supercoiled DNA (Waring, 1970; Wang, 1971), the studies on the binding of actinomycin D to DNA model polymers (Wells and Larson, 1970), the interaction of actinomycin D and the deoxydinucleotides (Krugh, 1972), and the work of Müller and coworkers with actinomycin C_3 (Schara and Müller, 1972; Zipper *et al.*, 1972).

The model of Sobell and Jain (1972) was based upon the crystal structure of an actinomycin D–deoxyguanosine complex (Sobell *et al.*, 1971; Sobell, 1973; Jain and Sobell, 1972) where they observed that actinomycin D cocrystallized with *two* deoxyguanosine molecules. We have recently reported (Krugh and Neely, 1973) a detailed proton magnetic resonance (pmr) investigation of the interaction of actinomycin D and several mononucleotides. We found that actinomycin D forms a complex with *two* deoxyguanosine 5′-monophosphate molecules in aqueous solution and that the geometry of the complex deduced from chemical shift changes in the actinomycin D resonances is consistent with the geometry observed in the solid state for an actinomycin D–deoxyguanosine complex (Sobell *et al.*, 1971).

The optical spectra (Krugh, 1972) also clearly demonstrated that actinomycin D exhibits a great deal of stereochemical specificity in forming complexes with deoxydinucleotides. From an analysis of the optical spectra we proposed a model for the interaction of actinomycin D with the various deoxynucleotides. The present results provide convincing support for this model.

Schara and Müller (1972) have recently reported the results of a study of the interaction of actinomycin C_3 with a series of deoxydinucleotides and an oligonucleotide. Actinomycin C_3 is similar to actinomycin D (C_1) except that the D-valine groups in the pentapeptide rings of actinomycin D are replaced by D-*allo*-isoleucine groups in actinomycin C_3 (Waksman, 1968). From optical spectral titrations and sedimentation equilibrium experiments Schara and Müller (1972) concluded that actinomycin C_3 forms a 1:2 complex with pdG-dC which agrees with our previous optical experiments (Krugh, 1972) and our present pmr experiments on actinomycin D. However, on the basis of sedimentation equilibrium experiments Schara and Müller concluded that actinomycin C_3 forms a 2:2 complex with pdC-dG. This is inconsistent with our present pmr results for the actinomycin D–pdC-dG complex where we observe the formation of a 1:2 complex. We feel there are several lines of evidence favoring the formation of the 1:2 complex, and we shall discuss these after the presentation of the results.

The complete assignment of the pmr spectra of actinomycin

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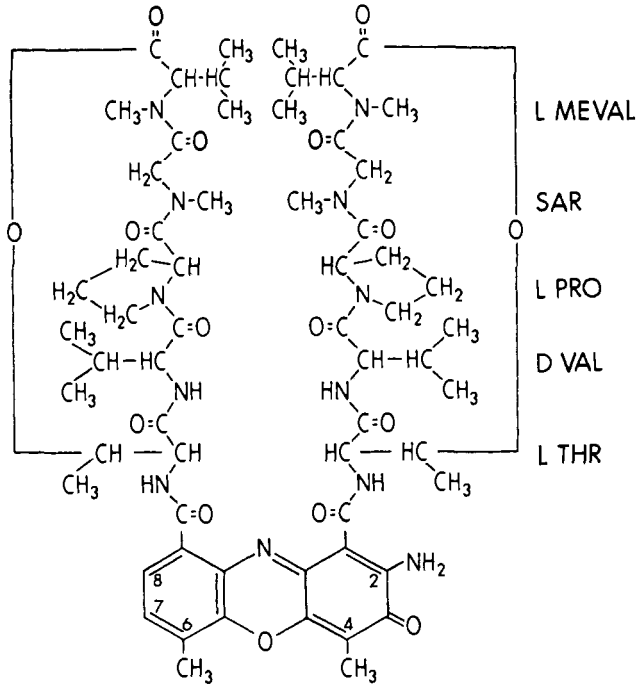

FIGURE 1: Structural formula of actinomycin D; abbreviations: Thr = threonine; Val = valine; Pro = proline; Sar = sarcosine; MeVal = methylvaline.

D in D_2O has been previously reported by Arison and Hoogsteen (1970) and Angerman *et al.* (1972) and these assignments have been used in the present study. At concentrations $\gtrsim 10^{-4}$ M actinomycin D tends to exist in the dimerized form (Crothers *et al.*, 1968). The dimerization process is an important consideration in the interpretation of the observed chemical shift changes during complex formation. In our previous paper (Krugh and Neely, 1973) on the pmr study of the actinomycin D–mononucleotide interactions we discussed the effect of dimerization and presented the results of a dilution experiment to determine the chemical shifts of the actinomycin D monomer. These experiments were performed under conditions appropriate to the present study and will be used in the interpretation of the present data.

Experimental Section

Actinomycin D was a gift of Merck Sharp & Dohme. A volatile impurity was removed by placing the actinomycin D under vacuum for 1 hr immediately prior to use. The deoxydinucleotides were purchased from Collaborative Research, Inc. The dinucleotides were also held under vacuum for 1 hr immediately prior to use. All samples were dissolved in a 5 mM potassium phosphate buffer in D_2O and the pD was adjusted with NaOD and DCl until the meter read 7.0. A weighed amount of actinomycin D was dissolved in cold (~4°) buffer to give ~0.02 M solutions. The actual concentration was determined spectrophotometrically using an ϵ_{425} of 23,500 (Smith, 1963). The concentrations of the deoxydinucleotides were also measured spectrophotometrically using extinction coefficients supplied by Collaborative Research, Inc. With the dinucleotides the concentrations determined spectrophotometrically occasionally differed from the values based upon the weights of the nucleotides and volumes of the solutions. We estimate that the concentrations of the dinucleotide solutions have an uncertainty of ±10%. The dinucleotides pdG-dC and pdC-dG dissolved in D_2O buffer to produce a

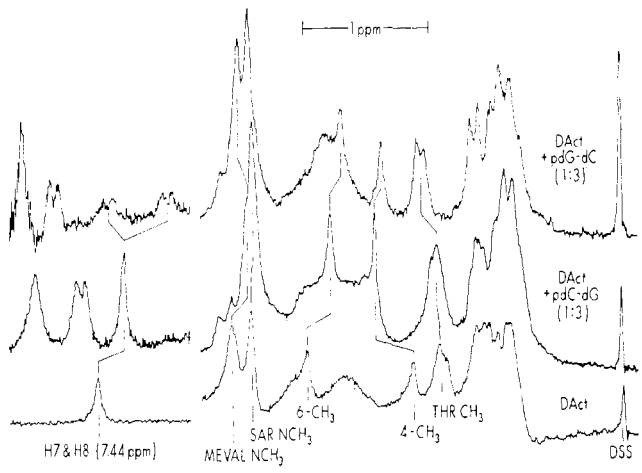

FIGURE 2: 100-MHz proton spectrum of (a) actinomycin D; (b) actinomycin D and pdC-dG in a 1:3 ratio; (c) actinomycin D and pdG-dC in a 1:3 ratio.

cloudy solution that precipitated upon standing. An alternate procedure was used in these cases. These dinucleotides were dissolved in D_2O and passed through a Millipore filter. The samples were then lyophilized and dissolved in the D_2O phosphate buffer and the pD was adjusted as before. This procedure produced a clear colorless solution. The concentration was measured and the appropriate aliquots placed in individual containers and frozen until needed. The nmr experiments were conducted continuously over an approximately 36-hr period with no visible precipitate forming in this period of time.

All reported chemical shifts were measured relative to the sodium salt of 2,2-dimethyl-2-silapentane-5-sulfonic acid. The concentration of the standard was kept as low as possible consistent with accurate chemical shift measurements (~4 mM at the beginning of each experiment.) We did not observe any influence of the reference compound on the pmr spectrum of actinomycin D or the nucleotides under these conditions. The uncertainties in the chemical shift measurements are estimated to be ±1 Hz in most cases. These uncertainties are generally indicated in the figures by the size of the symbol used to plot the titration curves (~2 Hz).

Results

pdG-dC and pdC-dG. All the deoxydinucleotides reported in this study will form complexes with actinomycin D. The composite spectra for the interaction of actinomycin D with the deoxydinucleotides pdG-dC and pdC-dG are shown in Figure 2. The solid lines between the spectra connect identical resonance lines. The H-7 and H-8 protons in the actinomycin D complex with pdG-dC are split into an AB pattern. In the actinomycin D complex with pdC-dG the H-7 and H-8 protons remain equivalent. Figure 3 shows the downfield portion of the spectrum for the incremental addition of pdG-dC to the actinomycin D solution for several nucleotide to drug ratios. The H-7 and H-8 initially appear to shift upfield and remain equivalent. At a 1:1 ratio the intensity of the singlet has diminished and the first indication of the doublet structure has appeared. At a 2:1 ratio the AB pattern has grown to full intensity and the singlet line is no longer observable. The 4-CH_3 and 6-CH_3 resonances in the actinomycin D titration with pdG-dC also broaden. These line broadenings and the behavior of the H-7 and H-8 protons are a result of chemical

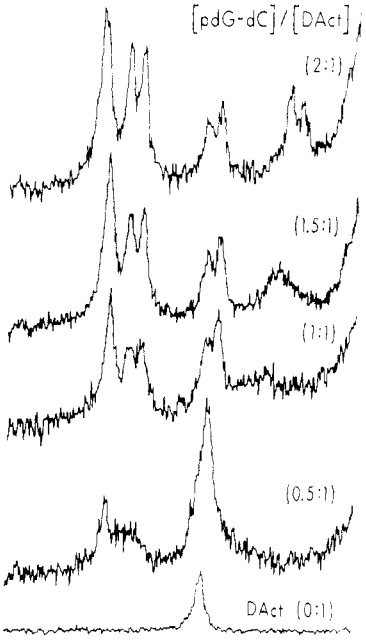

FIGURE 3: The H-7 and H-8 protons of actinomycin D for several nucleotide to drug ratios in the pdG-dC titration.

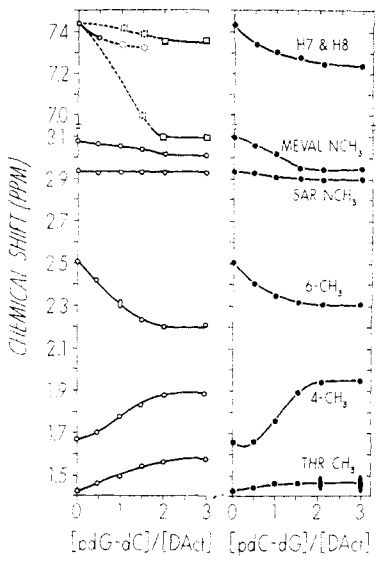

FIGURE 4: Chemical shifts of selected actinomycin D groups plotted as a function of the nucleotide to drug ratio for pdG-dC and pdC-dG.

exchange processes that occur at a rate comparable to the chemical shift differences between the various species participating in the equilibrium.[1] No comparable broadening of the 4-CH_3 and 6-CH_3 resonances has been observed in any other spectrum. Since no similar broadening was observed for other dinucleotides the rate of chemical exchange for the actinomycin D–(pdG-dC)$_2$ complex must be slower than in any other system.

The chemical shifts of several important actinomycin D groups are plotted as a function of the nucleotide to actinomycin D ratio for pdG-dC and pdC-dG in Figure 4. The difference in the various curves for the two dinucleotides again illustrates the sequence specificity of actinomycin D in its interaction with these dinucleotides. In all of the spectra we will be discussing the shapes of the titration curves and the induced chemical shifts (which we define as the change in the chemical shift of a particular resonance that results from an actinomycin D molecule forming a complex with the dinucleotides). In almost all the spectra the chemical shifts of the actinomycin D groups reach a limiting value after the nucleotide/actinomycin D ratio reaches a critical value of 2:1. The limiting chemical shifts are assumed to be the chemical shifts of the actinomycin D–dinucleotide complex since the addition of excess dinucleotide no longer influences the chemical shifts of the actinomycin D groups. The values of the limiting chemical shifts are used to calculate the induced shifts of the various actinomycin D groups in the following equilibrium

$$\text{actinomycin D} + n \text{ nucleotides} \underset{}{\overset{K}{\rightleftharpoons}} \text{actinomycin D–(nucleotide)}_n$$

The induced shifts are a function of the geometry of the complex and are very useful in the interpretation of the results.

However, actinomycin D exists predominantly as a dimer at the concentrations required for the pmr study and we must take this into account in the calculation of the induced shifts. The chemical shifts of the actinomycin D monomer may be determined from the concentration dependence of the actinomycin D chemical shifts by extrapolation to zero concentration (Krugh and Neely, 1973; Angerman *et al.*, 1972). The values for the induced shifts that result from complex formation of actinomycin D with the pdG-dC and pdC-dG dinucleotides are given in Table I. Also included are the induced shifts for the actinomycin D–(dGMP)$_2$ complex. These values show that the induced shifts for the dGMP (pdG) and pdC-dG complexes are similar. However, in comparing the induced shifts of the actinomycin D complexes with pdG-dC and pdC-dG the differences are clearly outside of experimental uncertainties. The uncertainties in the actinomycin D monomer shifts are not important in considering the induced shift of

TABLE I:[a] Induced Shifts of the Actinomycin D Protons That Result from Complex Formation with the Nucleotides.

	Nucleotide				
Resonance	pdG-dC	pdC-dG	dGMP[b]	pdT-dG	pdA-dG
4-CH_3	0.32	0.25	0.20	0.24	0.25
6-CH_3	0.46	0.36	0.34	0.37	0.37
H-7	0.71[c]				
and		0.36	0.32	0.31	0.35
H-8	0.25[c]				

[a] Values given are in ppm. The induced shift is the limiting chemical shift of the actinomycin D–nucleotide complex minus the actinomycin D monomer chemical shifts. Positive values indicate that the resonance position would move upfield for the complexation of an actinomycin D monomer molecule with the nucleotides. [b] These values are from Krugh and Neely (1973). [c] We have tentatively assigned the downfield resonance to the H-8 proton.

[1] The effect of chemical equilibrium or exchange processes on chemical shifts and line widths is treated by most general texts on nuclear magnetic resonance (*e.g.*, see Pople *et al.*, 1959, Jackman, 1969).

[2] The self-association of mono- and dinucleosides and -nucleotides has been the subject of a large number of investigations. The reader is referred to the following articles and references therein for an introduction to these studies: Ts'o *et al.*, 1969; Bangerter and Chan, 1969; Schweizer *et al.*, 1968.

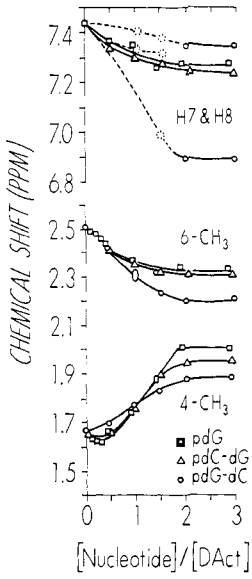

FIGURE 5: A composite drawing of the 4-CH_3, 6-CH_3, and H-7 and H-8 titration curves for the interaction of pdG, pdC-dG, and pdG-dC with actinomycin D.

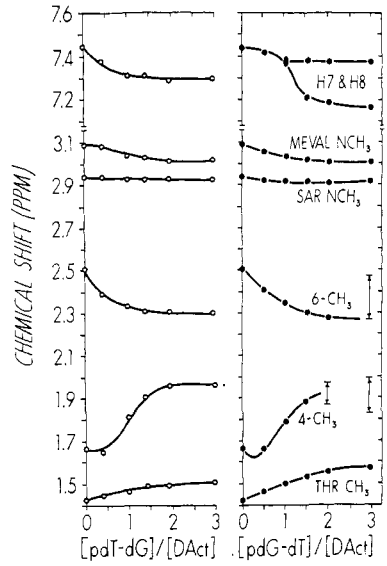

FIGURE 6: Chemical shifts of selected actinomycin D groups plotted as a function of the nucleotide to drug ratio for pdT-dG and pdG-dT.

each resonance for a series of nucleotides since the monomer shifts are essentially a fiducial point. However, these uncertainties in the actinomycin D monomer shifts are important in a comparison of the induced shifts of the various resonances (*e.g.*, comparing the induced shift of the 4-CH_3 group *vs.* the 6-CH_3 group).

In comparing the titration curves in Figure 4 we note that the most obvious difference is observed in the H-7 and H-8 protons. With pdG-dC the H-7 and H-8 protons split into an AB pattern. The H-7 and H-8 protons remain equivalent throughout the pdC-dG titration. The MeVal N-CH_3 group is shifted upfield less in the actinomycin D–$(pdG\text{-}dC)_2$ complex than in the actinomycin D–$(pdC\text{-}dG)_2$ complex. Conversely, the 6-CH_3 group is shifted upfield an additional 11 Hz in the actinomycin D–$(pdG\text{-}dC)_2$ complex compared to the limiting shift in the actinomycin D–$(pdC\text{-}dG)_2$ complex. The 4-CH_3 group in the actinomycin D–$(pdG\text{-}dC)_2$ complex is shifted upfield ~7 Hz more than in the actinomycin D–$(pdC\text{-}dG)_2$ complex. The threonine methyl groups are shifted downfield an additional ~10 Hz in the actinomycin D–$(pdG\text{-}dC)_2$ complex compared to the actinomycin D–$(pdC\text{-}dG)_2$ complex.

Additional information may be gained by considering the form of the curves in Figure 4. The analysis is not straightforward, however, since the chemical shifts are markedly influenced by the degree of dimerization of actinomycin D. The effect of dimerization is most easily seen in the behavior of the 4-CH_3 group. During the titration this group moves downfield even though the induced shift that results from complex formation of the nucleotides with an actinomycin D monomer produces an upfield shift of these actinomycin D resonances. The 4-CH_3 group has a net downfield movement because destacking the actinomycin D dimers produces a downfield shift (~0.53 ppm) that is larger than the induced upfield shift that results from complexation (for a more detailed discussion see Krugh and Neely, 1973). The shapes of the 4-CH_3 curves and the 6-CH_3 curves in Figure 4 are obviously different and reflect the different types of equilibria involved. The optical spectral titrations (Krugh, 1972) showed that actinomycin D complexes with two pdG-dC molecules in a cooperative fashion, while the titration of actinomycin D with pdC-dG produced a curve that was nearly hyperbolic, indicating the formation of a 1:1 complex or the presence of identical noninteracting sites. A composite drawing of the 4-CH_3 and 6-CH_3 curves for the interaction of actinomycin D with the nucleotides pdC-dG, pdG-dC, and 5′-dGMP is shown in Figure 5. The near linearity in both of the 4-CH_3 and 6-CH_3 curves in the pdG-dC titration and the behavior of the H-7 and H-8 resonances are consistent with the existence of a highly cooperative interaction in the formation of an actinomycin D–$(pdG\text{-}dC)_2$ complex. The values of the induced shifts listed in Table I are consistent with actinomycin D complexing with pdG-dC to form an intercalated type complex. The similarity of the titration curves (Figure 5) and the similarity of the induced shifts (Table I) for both the pdC-dG and 5′-dGMP complexes with actinomycin D strongly indicate that these two nucleotides are forming similar type complexes with actinomycin D.

The stoichiometry of the complexes may also be obtained from these spectra. In the pdG-dC titration the chemical shifts of the actinomycin D resonances are invariant above nucleotide to drug ratios of 2:1. In fact, the chemical shifts of the proton resonances cease changing rather abruptly when the solution contains twice as much pdG-dC as actinomycin D. Thus, we conclude that actinomycin D forms a complex with *two* pdG-dC nucleotides, which is consistent with the optical spectral results (Krugh, 1972). The chemical shifts of the actinomycin D protons in the titration with pdC-dG are also invariant above nucleotide/drug ratios >2:1 from which we conclude that this dinucleotide also forms a 2:1 complex with actinomycin D (*i.e.*, an actinomycin D–$(pdC\text{-}dG)_2$ complex).

pdT-dG and pdG-dT. The titration curves for the interaction of pdT-dG and pdG-dT with actinomycin D are shown in Figure 6. The most obvious difference between these sets of data is in the behavior of the H-7 and H-8 protons. In the pdT-dG titration the H-7 and H-8 protons remain equivalent while they split into an AB pattern during the pdG-dT titration. Spectral overlap of the thymidine resonances with the 4-CH_3 and 6-CH_3 groups resulted in rather large uncertainties in the limiting shifts of these groups in the pdG-dT titration, as indicated in Figure 6.

The chemical shifts of the various resonances in the pdT-dG

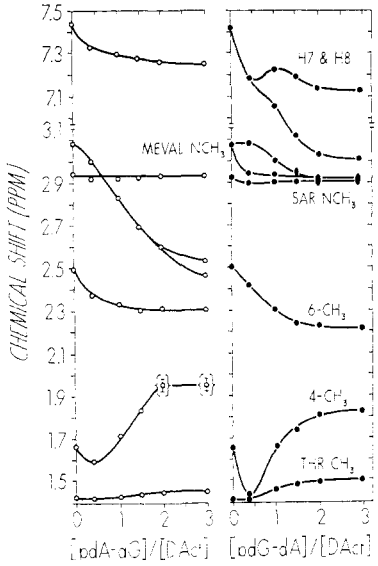

FIGURE 7: Chemical shifts of selected actinomycin D groups plotted as a function of the nucleotide to drug ratio for pdA-dG and pdG-dA.

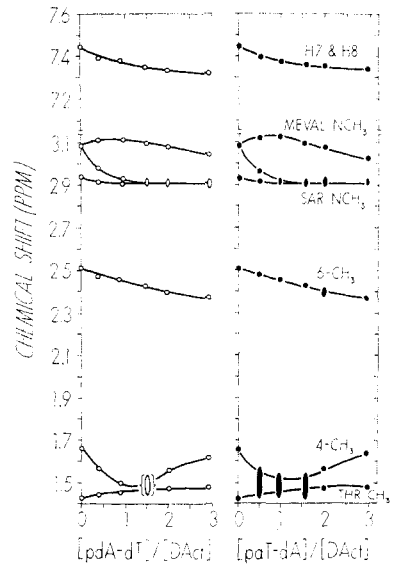

FIGURE 8: Chemical shifts of selected actinomycin D groups plotted as a function of the nucleotide to drug ratio for pdA-dT and pdT-dA.

titration are essentially invariant above nucleotide to actinomycin ratio >2:1. We thus conclude that pdT-dG complexes with actinomycin D to form an actinomycin D–(pdT-dG)$_2$ complex. The data are somewhat less conclusive for the pdG-dT titration but it appears that a 2:1 complex is also formed. The induced shifts for the actinomycin D–(pdT-dG)$_2$ complex are listed in Table I. The induced shifts for the 4-CH_3 group (0.24 ppm) and the 6-CH_3 group (0.37 ppm) are essentially the same as the values listed for the pdC-dG complex, and the pdA-dG complex. However, the induced shift of the H-7 and H-8 protons (0.31 ppm) is somewhat less than that observed for the pdC-dG complex (0.36 ppm) and the pdA-dG complex (0.35 ppm).

pdA-dG and pdG-dA. The data for the interaction of actinomycin D with pdA-dG and pdG-dA are shown in Figure 7. Even a cursory inspection of these data immediately illustrates that actinomycin D forms quite different complexes with these two dinucleotides. Once again the H-7 and H-8 protons split into an AB pattern with the addition of a dinucleotide of the form pdG-dN, where N is either C, T, or, in the present case, A. The H-7 and H-8 protons remain equivalent throughout the titration with pdA-dG, as observed in the other titrations with nucleotides of the form pdN-dG. The methylvaline N-CH_3 groups are strikingly different in the two titrations. During the pdA-dG titration the two methylvaline N-CH_3 are shifted upfield more than 0.5 ppm. The two methylvaline N-CH_3 groups become nonequivalent only when the [pdA-dG]/[actinomycin D] ratio was 3:1. During the pdG-dA titration the two methylvaline N-CH_3 groups are at first nonequivalent but then end up with the (approximately) same chemical shift at nucleotide/drug ratios $\gtrsim$1.5:1. In addition, the total upfield shift is less than 0.2 ppm. These data substantiate the model proposed earlier (Krugh, 1972) for the interaction of the dinucleotides as will be discussed below. The invariance of the chemical shifts of the 4-CH_3, the 6-CH_3, and the H-7 and H-8 resonances above nucleotide/drug ratios of 2:1 again indicates that actinomycin D forms a complex with *two* pdA-dG or *two* pdG-dA nucleotides.

pdA-dT and pdT-dA. The data for the interaction of pdA-dT and pdT-dA with actinomycin D are shown in Figure 8. Comparing these curves with those obtained for any of the dinucleotides containing guanine illustrates the differences in the type of interaction observed. The data in Figure 8 are virtually identical for both the pdA-dT and pdT-dA titrations and are significantly different from the curves observed for the actinomycin D–deoxyadenosine 5′-monophosphate titration reported previously (Krugh and Neely, 1973). The curves in Figure 8 do not appear to reach limiting values even at a nucleotide/drug ratio of 3:1. It is interesting to note that the H-7 and H-8 protons remain equivalent throughout both of these titrations. Unfortunately, these data are not amenable to direct analysis in terms of the stoichiometry or geometry of complex formation.

Discussion

Stoichiometry of Complex Formation. The incremental addition of mono- and dinucleotides to relatively concentrated solutions of actinomycin D has allowed observation of their interactions in solution by proton magnetic resonance. The chemical shifts of the 4-CH_3, 6-CH_3, and the H-7 and H-8 protons in the titrations of actinomycin D with pdG(5′-dGMP), pdN-dG, and pdG-dN (where N is A, T, or C) are invariant above nucleotide to actinomycin D ratios of ~2:1. These results provide convincing evidence that actinomycin D can interact in solution with two guanine residues. In other words, actinomycin D will form a complex with two guanine-containing dinucleotides.

This conclusion must be compared with the results from the optical spectral titrations, where the complex formation is monitored by observing the changes in the visible spectrum of actinomycin D after the addition of nucleosides or nucleotides (*e.g.*, Kersten, 1961; Reich, 1964; Behme and Cordes, 1965). The shape of the optical titration curve for 5′-dGMP is approximately hyperbolic which has been assumed to indicate the formation of a 1:1 complex. However, the optical experiments are not conclusive in that the presence of identical, noninteracting binding sites on actinomycin D will also result in a hyperbolic titration curve. A careful examination of the optical titration curves for the interaction of actinomycin D with pdC-dG and dGMP has indicated that these curves are not exactly hyperbolic (T. R. Krugh and M. N. Bresnick, 1972, un-

published results) which is consistent with the evidence that actinomycin D will complex with two guanine-containing nucleotides in aqueous solution. In an optical study of the interaction of actinomycin D and deoxydinucleotides (Krugh, 1972) we found that actinomycin D forms a complex with *two* pdG-dC molecules in a cooperative fashion. The present pmr experiments confirm this observation. Other evidence that actinomycin D forms a complex with more than one nucleoside is available from the crystal structure of an actinomycin D–deoxyguanosine complex where it was observed that actinomycin D cocrystallized with *two* deoxyguanosine molecules (Sobell *et al.*, 1971). Arison and Hoogsteen (1970) have also provided indirect evidence that actinomycin D forms a complex with two dGMP molecules. Schara and Müller (1972) concluded from sedimentation equilibrium experiments that actinomycin C_3 forms a complex with *two* pdG-dC molecules. This result is consistent with both our optical experiments and proton magnetic resonance experiments. On the other hand, they concluded that actinomycin D forms a 2:2 complex with pdC-dG. Actinomycin D and actinomycin C_3 differ only in one amino acid of each pentapeptide ring (Waksman, 1968) and both would be expected to form similar complexes with the dinucleotides. The proton magnetic resonance experiments, the optical experiments and the stoichiometry of the crystalline complex are all consistent with the conclusion that actinomycin D will bind *two* guanine containing nucleotides in solution.

Interactions with Guanine-Containing Nucleotides. The pmr chemical shift data for the 4-CH_3, the 6-CH_3 and the H-7 and H-8 protons for all the pdN-dG dinucleotides are quite similar to the data for pdG (Krugh and Neely, 1973). This indicates that all these nucleotides are forming similar type complexes with actinomycin D and that the interaction is predominantly with the guanine base. The geometry of these complexes is similar to the geometry deduced for the actinomycin D–dGMP complex (Krugh and Neely, 1973). The N bases in the complexes of the actinomycin D with the pdN-dG nucleotides must be located away from the phenoxazone ring since the N bases have very little effect on the binding (Krugh, 1972). The location of the N base is indicated by the chemical shifts of the methylvaline N-CH_3 groups in the titrations of actinomycin D with the pdN-dG dinucleotides. In the crystalline complex (Sobell *et al.*, 1971) the methylvaline N-CH_3 groups projected toward the plane of the phenoxazone ring in the area above the guanine bases. These groups are in a good position to monitor the environment immediately above the guanine rings. The increased upfield shift of the methylvaline N-CH_3 groups in the pdN-dG complexes as compared to the pdG complex with actinomycin D indicates that the N base tends to be located above the guanine ring. The magnitude of the induced shielding of the methylvaline N-CH_3 groups increases in the same order as the calculated ring currents in the bases (Giessner-Prettre and Pullman, 1970). The very large upfield shift of the methylvaline N-CH_3 groups in the pdA-dG complex with actinomycin D is probably a result of both the large ring currents of adenine and the fact that adenine has the greatest tendency to base stack with the guanine ring (*e.g.*, see the references in footnote 2 or Giessner-Prettre and Pullman, 1970).

The two methylvaline N-CH_3 groups have the same chemical shift throughout the titrations with the pdG and pdN-dG nucleotides. The two threonine CH_3 groups also remain equivalent throughout these titrations. This indicates that dinucleotides of the form pdN-dG bind to the two binding sites of actinomycin D with similar geometries and similar binding constants. If the geometrical relationship between the methylvaline N-CH_3 or the threonine CH_3 and the bound nucleotides was significantly different in the two binding sites, then we would expect that the limiting chemical shifts of these groups would be different. Since the two groups remain equivalent during the entire titration, the two binding sites must be occupied to a similar extent. The methylvaline N-CH_3 resonances in the pdA-dG titration continue to move upfield and become nonequivalent above a nucleotide to drug ratio of 2:1. The reason for this is not well understood but it is most likely due to nonspecific base stacking of the excess nucleotide with the actinomycin D–$(pdA\text{-}dG)_2$ complex.

We have also undertaken a theoretical analysis of the titration curves to determine if the shapes of the pmr titration curves are in agreement with the optical spectral results. As expected, the pdN-dG titration curves can be reproduced by assuming that actinomycin D has two independent binding sites and that the pdN-dG nucleotides have a slight preference for the binding site on the benzenoid portion of the chromophore.

Dinucleotides of the form pdG-dN produce distinctly different chemical shift changes in the spectrum of actinomycin D than the pdN-dG dinucleotides. Actinomycin D tends to form geometrically specific complexes in solution with dinucleotides containing guanine, consistent with the observation that the guanine base is the structural determining factor. In the titration of actinomycin D with pdG-dC we observed several distinct characteristics. The shapes of the curves for the Thr-CH_3, the 4-CH_3 and the 6-CH_3 groups were quite different from those observed with the pdC-dG and pdG titrations (Figure 5). Our theoretical analysis of the pdG-dC binding to actinomycin D reproduces the experimental curve if we assume that actinomycin D forms a complex with *two* pdG-dC dinucleotides in a highly cooperative fashion, in agreement with the optical titration data (Krugh, 1972). The magnitudes of the induced upfield shifts of the 4-CH_3 and the 6-CH_3 groups listed in Table I show that the actinomycin D–$(pdG\text{-}dC)_2$ complex has the largest induced shifts. The magnitudes of the induced shifts for the actinomycin D–$(pdG\text{-}dC)_2$ complex are consistent with this complex having a geometry in which the two pdG-dC dinucleotides are base paired to one another with the phenoxazone ring intercalated between the two G·C base pairs. The additional induced upfield shielding of the 4-CH_3 and the 6-CH_3 groups, as compared to the pdG complex, is due to the presence of the cytosine rings. In the intercalated type complex these two rings will be located near the 4-CH_3 and the 6-CH_3 groups, respectively.

Of all the nucleotides studied, the complex of actinomycin D with pdG-dC is the only one for which we observed significant line broadening due to slow chemical exchange processes (Figure 3). This is consistent with our earlier observation that actinomycin D binds the pdG-dC dinucleotide in a cooperative manner (Krugh, 1972). The strong affinity of the actinomycin D for the pdG-dC dinucleotide presumably slows the exchange rate between free and bound actinomycin D so that line broadening is observed during the titration. The complex of actinomycin D with the pdG-dC dinucleotide is thus the most long-lived complex of any studied. This is consistent with this dinucleotide forming an intercalated type complex with actinomycin D as proposed from the optical spectra (Krugh, 1972). Watson–Crick base pairing of the self-complementary dinucleotide predicts the formation of six hydrogen bonds which would tend to stabilize the complex. A consideration of the H-7 and H-8 resonances in the pdG-dC and pdC-dG complexes with actinomycin D is sufficient to

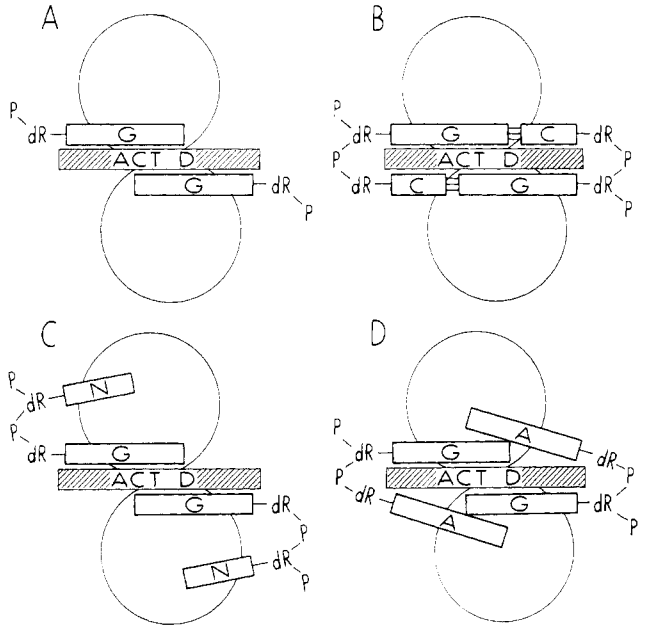

FIGURE 9: Schematic illustrations of actinomycin D complexes. In these figures the phenoxazone ring projects out of the plane of the paper and is indicated by the dashed lines. The cyclic pentapeptide rings are schematically illustrated as circles. (A) Actinomycin D–deoxyguanosine 5′-monophosphate complex; (B) actinomycin D–pdG-dC complex, illustrating the formation of an intercalated complex: (C) actinomycin D–pdN-dG complex; (D) actinomycin D–pdG-dA complex, illustrating steric interference of the noncomplementary bases.

illustrate the sequence specificity of actinomycin D. Both of these dinucleotides are self-complementary and in theory both should be able to form intercalated type complexes with actinomycin D. Of these two dinucleotides only pdG-dC appears to form an intercalated complex with actinomycin D.

In the titrations of actinomycin D with the pdN-dG dinucleotides the H-7 and H-8 protons remain equivalent, while in the pdG-dN titrations (N = A, C, T) the H-7 and H-8 protons split into an AB pattern. Most likely the H-7 and H-8 protons split into the AB pattern because the phosphodiester linkage of one of the pdG-dN dinucleotides is in the vicinity of one of these protons. Similar behavior was noted for the interaction of actinomycin D with GMP where the presence of the 2′-hydroxyl group caused the H-7 and H-8 protons to split into an AB pattern as the complex was formed (Krugh and Neely, 1973).

The methylvaline N-CH_3 groups do not remain equivalent during the pdG-dA titration (Figure 7), although they both have approximately the same limiting shift. This is an indication that the two binding sites have significantly different binding constants for pdG-dA. These data must be interpreted carefully when referring to binding constants because the original actinomycin D solution is predominantly in the dimer form at the concentrations (~0.02 M) used for the pmr experiments (Crothers *et al.*, 1968; Angerman *et al.*, 1972; Krugh and Neely, 1973). The dinucleotides may bind to the dimerized form of actinomycin D and this may affect the apparent constants.

The large initial upfield shift of the 4-CH_3 group is a distinctive feature of the pdG-dA dinucleotide titration. The actinomycin D dimer results from a stacking of the phenoxazone rings with one chromophore inverted with respect to the other (Angerman *et al.*, 1972). A consideration of molecular models shows that pdG-dA could bind to the actinomycin D dimer with the guanine end bound to one actinomycin D phenoxazone ring in the normal manner. In addition, the adenine ring can be associated with the *other* phenoxazone ring resulting in a sandwich type complex with the actinomycin D dimer in the middle. This could account for the initial upfield shift of the 4-CH_3 being larger than that for the 6-CH_3 since the ring currents in adenine are larger than those for guanine. The data indicate that this type of complex can only exist at very low nucleotide to actinomycin D ratios since the 4-CH_3 groups begin to move downfield when the nucleotide to drug ratio is larger than 0.5:1.

Proposed Model. Schematic representations of the interactions of the nucleotides with actinomycin D are shown in Figure 9. The actinomycin D molecule contains two cyclic pentapeptide groups as indicated by the circles in Figure 9. The approximate twofold symmetry of the molecule is evident by the representation in Figure 9. As shown in this figure the two guanine bases in all of the nucleotides are bound to the actinomycin D with the same relative geometry. The induced shifts of the 4-CH_3, the 6-CH_3 and the H-7 and H-8 protons for all the nucleotides are consistent with this proposal. The location of the N base is thus determined by the sequence of the bound nucleotide. In the pdG-dN series the N bases tend to wrap around the phenoxazone ring, while in the pdN-dG series the N bases tend to base stack with the guanine ring. This interpretation is entirely consistent with the data. Since the nature of the N base has little influence on the titration curves of the 4-CH_3, the 6-CH_3, and the H-7 and H-8 protons for the pdN-dG series we conclude that this base is not adjacent to the phenoxazone ring. The N base should influence the chemical shifts of the peptide protons in the pdN-dG series. We observed that the induced shift of the methylvaline N-CH_3 group was proportional to the magnitude of the ring current in the N base, as predicted by our model. However, the N base does affect the chemical shifts of the 4-CH_3, the 6-CH_3, and the H-7 and H-8 protons in the pdG-dN series. This is also consistent with our model (Figure 9) since the N base will have a tendency to stack on the opposite side of the phenoxazone ring. With the self-complementary pdG-dC dinucleotide the formation of G·C base pairs results in the complementary interaction of this nucleotide with actinomycin D to form an intercalated complex. With pdG-dA or pdG-dT steric interference is expected (Figure 9D) since these dinucleotides are not self-complementary. The data do indicate that actinomycin D will bind *two* pdG-dA and pdG-dT molecules under the experimental conditions used for the nuclear magnetic resonance experiments.

The model proposed here for the interactions of actinomycin D and the deoxydinucleotides is consistent with both the visible spectroscopic results (Krugh, 1972) and the proton magnetic resonance experiments reported here and previously (Krugh and Neely, 1973).

Biochemical Implications. The pmr spectra confirm the previously observed specificity of the interaction of actinomycin D with the dinucleotides containing guanine (Krugh, 1972). The complex of actinomycin D and the self-complementary dinucleotide pdG-dC results in the formation of an intercalated type complex. The induced chemical shift changes in the 4-CH_3 and the 6-CH_3 groups are consistent with this complex having a geometry similar to that proposed in the model of Sobell and Jain (1972) for the interaction of the drug with DNA. In the crystalline complex of actinomycin D and deoxyguanosine strong hydrogen bonds (2.8 Å) were formed between the guanine 2-amino groups and the carbonyl oxygens

of the L-threonine residues while weaker hydrogen bonds (3.2 Å) were formed between the guanine N (3) ring nitrogen and the NH groups on the same L-threonine residues (Sobell *et al.*, 1971). These hydrogen bonds undoubtedly contribute to the stability of the complex. The formation of an intercalated-type complex between actinomycin D and the self-complementary dinucleotide pdG-dC predicts the formation of *six* additional hydrogen bonds from the two G·C base pairs. The formation of the G·C base pairs was assumed to be the source of the highly cooperative binding of the pdG-dC to actinomycin D observed in the optical spectra (Krugh, 1972). The complex formed between actinomycin D and the self-complementary dinucleotide pdC-dG would have six hydrogen bonds if it formed an intercalated type complex (the stereochemistry of the complex would not allow the formation of the four hydrogen bonds between the two guanine rings and the threonine residues). If the actinomycin D forms a complex with pdC-dG as pictured in Figure 9, only four hydrogen bonds are formed (the same four as discussed above). If the formation of hydrogen bonds is the primary consideration in determining the type of complex formed then we would predict that both pdG-dC and pdC-dG would form intercalated-type complexes with actinomycin D. Since the experiments clearly show that pdC-dG predominantly does not form an intercalated-type complex we conclude that hydrogen-bond formation is not the dominant factor in determining the type of complex formed. The nature of the forces that stabilize these complexes is of great interest since these complexes appear to serve as good models for the interaction of actinomycin D with DNA.

The pmr experiments also provide additional insight into the actinomycin D–DNA interaction. These experiments provide additional evidence that actinomycin D has a preference for the G-C sequence of DNA. This specificity was observed in the binding studies of Wells and Larson (1970) and predicted by the model by Sobell and Jain (1972). Actinomycin D will bind to other sequences of DNA with a general requirement for the presence of a deoxyguanosine residue (Wells and Larson, 1970; Goldberg *et al.*, 1962). The two nucleotide binding sites on actinomycin D appear to have approximately equal binding constants for dGMP. However, dAMP appears to bind to the benzenoid portion of the chromophore much stronger than it binds to the quinoid portion of the chromophore (Krugh and Neely, 1973). The quinoid portion of the chromophore appears to be more selective in its interactions with the DNA bases. We suggest that this selectivity is the origin of the preference for guanine in the binding of the drug to DNA. Of course, there are also other factors that influence the binding (*e.g.*, primary and secondary structure of DNA in the region of binding) and further experiments are required to determine the relative importance of each of these.

The use of deoxydinucleotides as models to study drug–DNA interactions in general appears to be very promising. The optical experiments (Krugh, 1972) and the present results have demonstrated that deoxydinucleotides, especially the self-complementary deoxydinucleotides, can be used to model the actinomycin D–DNA interaction. We have also studied the interaction of ethidium bromide with the deoxydinucleotides (Krugh *et al.*, manuscript in preparation). Ethidium bromide is a much simpler molecule than actinomycin D and yet we have found that this molecule also exhibits a specificity in forming complexes with the self-complementary deoxydinucleotides. This observation of sequence specificity for the interaction of ethidium bromide with the deoxydinucleotides undoubtedly reflects the specificity with which the drug binds to DNA. It will certainly be of interest to see if sequence specificity is a general phenomenon. This work also suggests that oligonucleotides will also be very useful in studying drug–DNA interactions. Self-complementary oligonucleotides may also prove to be very useful in determining the nature of the interactions between proteins, such as repressor molecules, with the nucleic acids.

References

Angerman, N. S., Victor, T. A., Bell, C. L., and Danyluk, S. S. (1972), *Biochemistry 11*, 2402.

Arison, B. H., and Hoogsteen, K. (1970), *Biochemistry 9*, 3976.

Bangerter, B. W., and Chan, S. I. (1969), *J. Amer. Chem. Soc. 91*, 3910.

Behme, M. T. A., and Cordes, E. H. (1965), *Biochim. Biophys. Acta 108*, 312.

Crothers, D. M., Sabol, S. L., Ratner, D. I., and Müller, W. (1968), *Biochemistry 7*, 1817.

Giessner-Prettre, C., and Pullman, B. (1970), *J. Theor. Biol. 27*, 87.

Goldberg, I. H., and Friedman, P. A. (1971), *Annu. Rev. Biochem. 40*, 772.

Goldberg, I. H., Rabinowitz, M., and Reich, E. (1962), *Proc. Nat. Acad. Sci. U. S. 48*, 2094.

Jackman, L. M. (1969), Applications of Nuclear Magnetic Resonance in Organic Chemistry, 2nd ed, New York, N. Y., Pergamon Press.

Jain, S. C., and Sobell, H. M. (1972), *J. Mol. Biol. 68*, 1.

Kersten, W. (1961), *Biochim. Biophys. Acta 47*, 610.

Kersten, W., Kersten, H., and Rauen, H. M. (1960), *Nature (London) 187*, 60.

Kirk, J. (1960), *Biochim. Biophys. Acta 42*, 167.

Krugh, T. R. (1972), *Proc. Nat. Acad. Sci. U. S. 69*, 1911.

Krugh, T. R., and Neely, J. W. (1973), *Biochemistry 12*, 1775.

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

Pople, J., Schneider, W. G., and Bernstein, H. J. (1959), High Resolution Nuclear Magnetic Resonance, New York, N. Y., McGraw Hill.

Reich, E. (1964), *Science 143*, 684.

Reich, E., Franklin, R. M., Shatkin, A. J., and Tatum, E. L. (1961), *Science 134*, 556.

Reich, E., and Goldberg, I. H. (1964), *Progr. Nucl. Acid Res. 3*, 183.

Schara, R., and Müller, W. (1972), *Eur. J. Biochem. 29*, 210.

Schweizer, M. P., Broom, A. D., Ts'o, P. O. P., and Hollis, D. P. (1968), *J. Amer. Chem. Soc. 90*, 1042.

Smith, M. (1963), *Biochem. Biophys. Res. Commun. 10*, 67.

Sobell, H. M. (1973), *Progr. Nucl. Acid. Res. 13*, 153.

Sobell, H. M., and Jain, S. C. (1972), *J. Mol. Biol. 68*, 21.

Sobell, H. M., Jain, S. C., Sakore, T. D., and Nordman, C. E. (1971), *Nature (London) 231*, 200.

Ts'o, P. O. P., Kondo, N. S., Schweizer, M. P., and Hollis, D. P. (1969), *Biochemistry 8*, 997.

Waksman, S. A., Ed. (1968), Actinomycin, New York, N. Y., Interscience Publishers, p 39.

Wang, J. C. (1971), *Biochim. Biophys. Acta 232*, 246.

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

Wells, R. D., and Larson, J. E. (1970), *J. Mol. Biol. 49*, 913.

Zipper, P., Kratry, O., Bunemann, H., and Müller, W. (1972), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 25*, 123.