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# Proton Magnetic Resonance Studies of Actinomycin D Complexes with Mixtures of Nucleotides as Models for the Binding of the Drug to DNA†

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ABSTRACT: The proton magnetic resonance spectra of actinomycin solutions with mixtures of deoxynucleotides have been investigated to determine the relative preference for the binding of guanine and adenine nucleotides to the two nucleotide binding sites of actinomycin D. An analysis of the chemical shifts of the actinomycin D resonances shows that adenine and guanine nucleotides competitively bind to the benzenoid portion of the phenoxazone ring of actinomycin D while guanine nucleotides bind stronger than adenine nucleotides to the quinoid portion of the phenoxazone ring. The chemical shift data for the titrations of actinomycin D with pdG-dG, pdC-dC, and an equimolar mixture of these complementary deoxydinucleotides show that: (1) pdG-dG forms a stacked complex much like dGMP; (2) pdC-dC does not bind to actinomycin D under the conditions used in these experiments; (3) in the titration of actinomycin D with the equimolar mixture of pdG-dG + pdC-dC, a miniature intercalated complex is formed.

The binding of actinomycin D to DNA has been studied by a variety of techniques over the past 15 years (e.g., see the reviews by Hollstein, 1974; Meienhofer and Atherton, 1973; and the many references therein). Müller and Crothers (1968) proposed that the phenoxazone ring of actinomycin D (Figure 1) intercalates into the DNA double helix, with the two cyclic pentapeptides located in the minor groove of the helix. The intercalation model for actinomycin D binding to DNA was supported by the data of Waring (1970) and Wang (1971). When actinomycin D binds to DNA, there is a general, but not absolute, requirement for a guanine base at the intercalation site (e.g., see Wells and Larson, 1970). Sobell et al. (1971) determined the three-dimensional structure of an actinomycin D-deoxyguanosine (1:2) crystalline complex, which was used to construct a detailed molecular model for the intercalation of actinomycin D at a dG-dC sequence of DNA (Sobell and Jain, 1972; Sobell, 1973). The complexation of actinomycin D to DNA involves base stacking, hydrogen bonding, and hydrophobic forces and thus a detailed understanding of the complex formation requires a knowledge of the relative contributions of each of these forces to the stabilization of the complex. The question of the origin of the guanine requirement is also of great interest. In previous nuclear magnetic resonance (NMR)[1] and optical titrations (Krugh, 1972; Krugh and Neely, 1973 a,b; Krugh and Chen, 1975), we have established that actinomycin D will bind two guanine containing deoxydinucleotides (see also Schara and Müller, 1972) or two dGMP molecules (see also Arison and Hoogsteen, 1970; Danyluk and Victor, 1970; Patel, 1974). The NMR titrations provide an opportunity of monitoring the binding of the nucleotides to the individual binding sites on the phenoxazone ring of actinomycin D. An actinomycin D titration with dAMP (Krugh and Neely, 1973a; Krugh and Chen, 1975) suggested that dAMP binds almost as strongly as dGMP to the benzenoid portion of the phenoxazone ring and much weaker than dGMP at the quinoid portion of the phenoxazone ring. The present manuscript and the following manuscript (Chiao and Krugh, 1977) explore the preferential binding of the nucleotides to actinomycin D as a model for the binding of the drug to DNA.

## Experimental Section

The deoxydinucleotides were purchased from Collaborative Research, Inc., and were used without further purification. A few of the samples were either treated with Chelex-100 (Bio-Rad) or a small amount of EDTA (ethylenediaminetetraacetic acid, sodium form) was added if there was appreciable line broadening observed in the dinucleotide proton magnetic resonance spectra. The dinucleotides were weighed into a sample tube and dissolved in 5 mM $D_2O$ phosphate buffer to yield a concentration in the 50 to 80 mM concentration range. The pD was then adjusted using DCl or NaOD to give a pH meter reading of 7.0. The concentration of the dinucleotides

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[1]Abbreviations used: NMR, nuclear magnetic resonance; EDTA, ethylenediaminetetracetic acid; TSP, sodium 3-trimethylsilylpropionate-*2,2,3,3-d*$_4$.

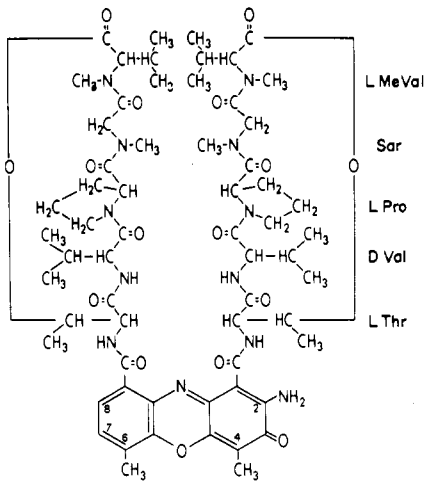


FIGURE 1: Structural formula of actinomycin D. Abbreviations used are: Thr, threonine; Val, valine; Pro, proline; Sar, sarcosine; MeVal, methylvaline.

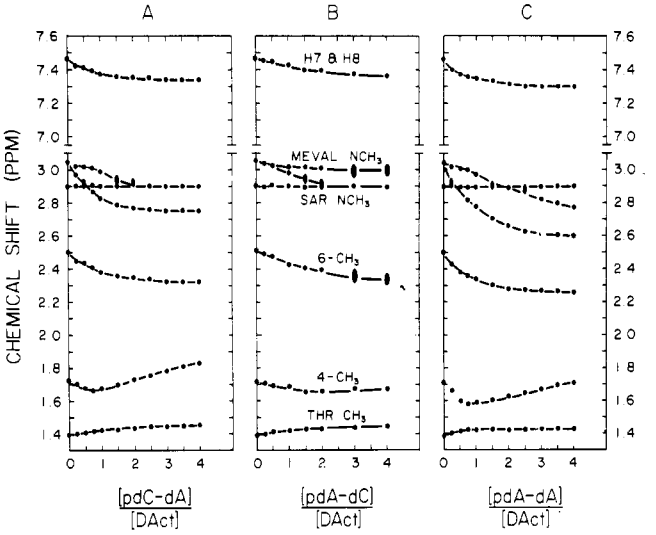


FIGURE 3: Chemical shifts of selected actinomycin D groups plotted as a function of the nucleotide/drug ratio for (A) pdC-dA; (B) pdA-dC; (C) pdA-dA. The spectra were recorded at 6 °C, 5 mM actinomycin D.

was determined at 260 nm using either the extinction coefficients supplied by Collaborative Research or the values listed in P-L Biochemicals Catalog No. 103.

The actinomycin D (a gift of Merck Sharp and Dohme) was placed under vacuum for several hours prior to use in order to remove any residual methanol. Approximate concentrations were determined by weighing the actinomycin D, followed by dissolution into a known volume of cold $D_2O$ phosphate buffer. The pD was adjusted as before and the concentration was determined spectrophotometrically using $\epsilon_{425} = 23{,}500$ (Hyman and Davidson, 1971). The concentration used was approximately 5 mM at the beginning of each titration.

The titrations were performed by placing 250 $\mu$l of cold (6 °C) actinomycin D stock solution into a standard 5-mm NMR tube. One microliter of 0.01 M TSP (sodium 3-trimethylsilylpropionate-*2,2,3,3-d*$_4$, Merck) was added to serve as an internal reference. An appropriate volume of the nucleotide solution was then added with a Hamilton microliter syringe and the solution was thoroughly mixed. In the titrations using nucleotide mixtures, the two nucleotides were mixed in the correct proportion before addition to the actinomycin D solution. The $^1$H NMR spectra were recorded after each addition on a JEOL PFT-100 Fourier transform spectrometer. Each spectrum required between 50 and 300 accumulations to enhance the signal-to-noise ratio. The temperature was controlled with the JEOL VES-VT-3 temperature regulator and was determined from the chemical shift of methanol (Van Geet, 1970). The final spectrum in each titration was also recorded at ambient temperature (~25 °C) where the smaller line widths made the observation of small splittings more readily observable.

## Results

Before considering the movement of the chemical shifts of the actinomycin D protons as a function of added nucleotides, it is important to note that, under these experimental conditions, actinomycin D exists predominantly as a dimer at the start of the titration (e.g., see Krugh and Chen, 1975; Crothers et al., 1968; Angerman et al., 1972). We have previously demonstrated that the limiting chemical shifts (i.e., the chemical shifts of the actinomycin D protons in the presence of excess dinucleotide) are independent of the original aggregation of the actinomycin D (Krugh and Neely, 1973a,b; Krugh and Chen, 1975). The infinite dilution chemical shifts of the actinomycin D groups are: 2.26 ppm (4-$CH_3$); 2.65 ppm (6-$CH_3$); and 7.52 ppm (H(7) and H(8)). From the limiting chemical shifts, we calculate that the 4-$CH_3$ resonance is shifted upfield +0.44 ppm, while the 6-$CH_3$ resonance is shifted upfield +0.50 ppm as a result of complex formation of actinomycin D with two dG-dC molecules. These upfield shifts are indicative of the formation of an intercalated complex in which the nucleotide bases are stacked on the phenoxazone ring of actinomycin D. The H(7) and H(8) protons have the same chemical shift in the actinomycin D spectrum and thus only a single resonance is observed. In the actinomycin D–2 (dG-dC) spectrum, the H(7) and H(8) protons have different chemical shifts and thus a "doublet of doublets" (called an AB pattern) is observed. We assume that the appearance of the AB pattern results from the magnetic anisotropy of the phosphate oxygen in the immediate vicinity of the H(7) and H(8) protons (e.g., see Krugh and Neely, 1973b; Krugh and Chen, 1975, for a discussion). Thus the observation of an AB pattern for the H(7) and H(8) protons serves as a reliable indication that the deoxydinucleotide is wrapped around the benzenoid portion of the phenoxazone ring of actinomycin D. This is clearly illustrated by comparing the data for the titration of actinomycin D with pdG-dC as opposed to pdC-dG or 5′-dGMP (Krugh and Neely, 1973a,b). The changes in the proton chemical shifts of the key actinomycin D groups in the actinomycin D titration with pdC-dG or 5′-dGMP are consistent with the formation of the stacked complexes illustrated in Figure 2B (see paragraph at the end of this paper concerning supplementary material). On the other hand, actinomycin D forms an intercalated complex with two pdG-dC molecules (Figure 2A).

The purpose of this study is to investigate the complexes of actinomycin D with binary mixtures of deoxynucleotides. The equilibria are fairly complex in these experiments as there may be as many as eight different kinds of complexes in simultaneous equilibrium during the titrations. As a result, we will concentrate our attention on the spectra observed at or near the end of the titration where the nucleotides are present in excess (on a molar basis).

*Actinomycin D Titrations with pdC-dA, pdA-dC, and pdA-dA.* The $^1$H NMR titrations of actinomycin D with pdC-dA, pdA-dC, and pdA-dA are shown in Figure 3. The H(7) and H(8) proton resonance remains a singlet throughout

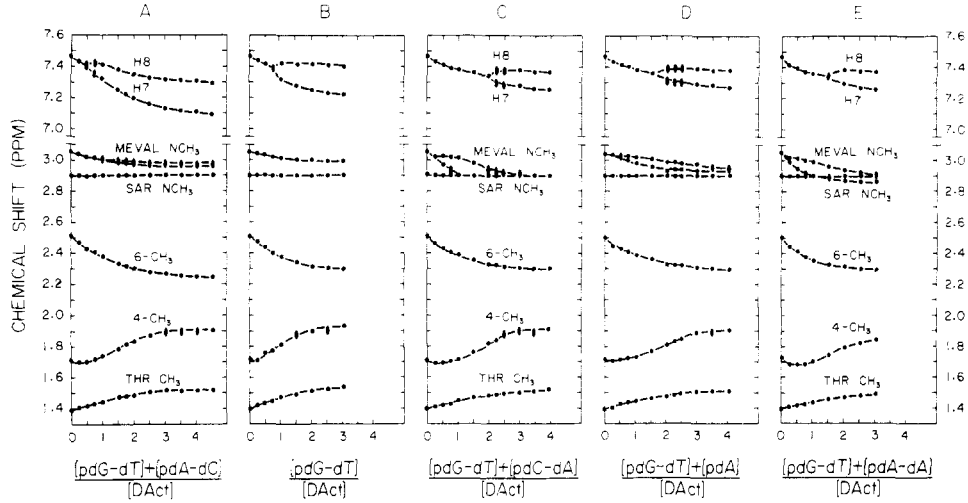


FIGURE 4: Chemical shifts of selected actinomycin D groups plotted as a function of the (total nucleotide)/drug ratio for (A) an equimolar mixture of pdG-dT and pdA-dC; (B) pdG-dT; (C) an equimolar mixture of pdG-dT and pdC-dA; (D) an equimolar mixture of pdG-dT and pdA; (E) an equimolar mixture of pdG-dT and pdA-dA. The spectra were recorded at 6 °C, 5 mM actinomycin D.

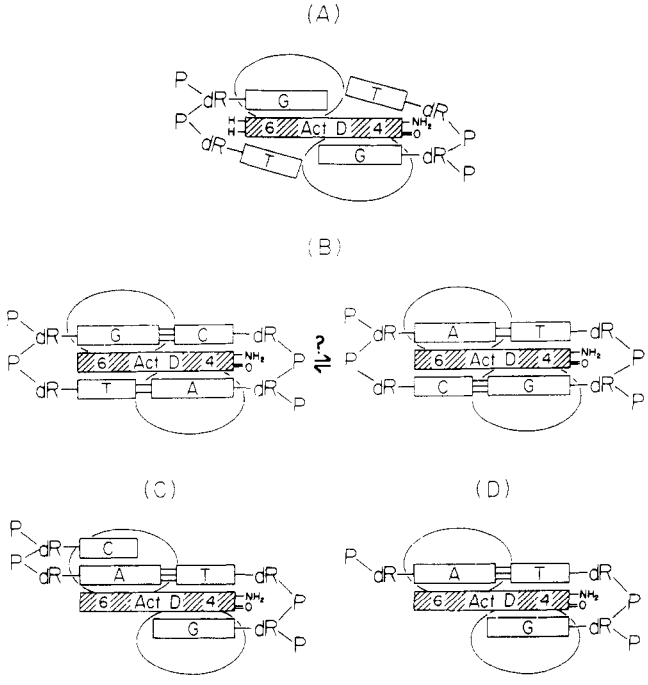


FIGURE 6: Schematic illustrations of actinomycin D complexes. (A) The Act D-2 (pdG-dT) complex, illustrating steric interference of the noncomplementary bases; (B) two distinct intercalated complexes with the mixture of pdG-dT and pdA-dC; (C) the Act D-pdG-dT-pdC-dA complex; (D) the Act D-pdA-pdG-dT complex.

these titrations, which indicates that these deoxydinucleotides do not tend to tightly "wrap around" the benzenoid portion of the phenoxazone ring. The two methylvaline $NCH_3$ groups lose their degeneracy in all three titrations which is an indication that different types of complexes are formed at the 4- and 6-binding sites. In all three of these titrations (Figure 3), the magnitude of the ring current induced shifts of the H(7) and H(8) resonance (0.15–0.22 ppm) and the 6-$CH_3$ resonance (0.3–0.4 ppm) shows that an adenine ring is stacked on the phenoxazone ring in the vicinity of these protons. As a result of both the weak binding of these dinucleotides at the 4-binding site and the large dimerization shift of the 4-$CH_3$ group, the chemical shift data for the 4-$CH_3$ groups are much less informative than the data for the other actinomycin D groups in Figure 3.

*Actinomycin D Titrations with Binary Mixtures Involving pdG-dT.* The titrations of actinomycin D with pdG-dT as well as binary mixtures of nucleotides involving pdG-dT are shown in Figure 4. In the pdG-dT titration (Figure 4B), the H(7) and H(8) protons split into an AB pattern (Figure 5 in supplementary material), which is indicative of the formation of a complex in which a pdG-dT molecule is wrapped around the benzenoid portion of the chromophore. A schematic diagram of this complex is shown in Figure 6A. The chemical shifts of the 4-$CH_3$ and 6-$CH_3$ groups are consistent with this interpretation (see Krugh and Neely, 1973b, for a more detailed discussion).

The titration of actinomycin D with the mixture of complementary deoxydinucleotides pdG-dT + pdA-dC is shown in Figure 4A. The H(7) and H(8) protons split into an AB pattern (Figure 5 in supplementary material) very early in the titration, which is indicative of the formation of a complex in which a deoxydinucleotide is wrapped around the benzenoid portion of the phenoxazone ring. The limiting chemical shifts of both the 4-$CH_3$ and the 6-$CH_3$ resonances in this titration are slightly upfield of the chemical shifts of these groups in the 5′-dGMP titration, which is consistent with the formation of an intercalated complex because base pair formation will result in an additional shielding of the 4-$CH_3$ and 6-$CH_3$ groups by the pyrimidine bases stacked on the phenoxazone ring. This additional shielding is expected to be relatively small (on the order of 5–10 Hz) since the ring currents of the pyrimidine bases are smaller than the ring currents of the purine bases (Giessner-Prettre and Pullman, 1976). A comparison of the behavior of the methylvaline $NCH_3$ groups in the pdG-dT and the pdG-dT + pdA-dC titrations (Figures 4A and 4B) also reflects the change in the structure of the complex formed when the complementary deoxdinucleotide is present.

Actinomycin D can form two distinct intercalated complexes with the complementary mixture pdG-dT + pdA-dC as schematically illustrated in Figure 6B. These two complexes are distinct because the phenoxazone ring of actinomycin D is asymmetric. It is an interesting and important question as to whether these complexes exist in equal proportions (i.e., a 50:50 mixture) or whether one of the complexes predominates in solution. The present titrations are not definitive with respect

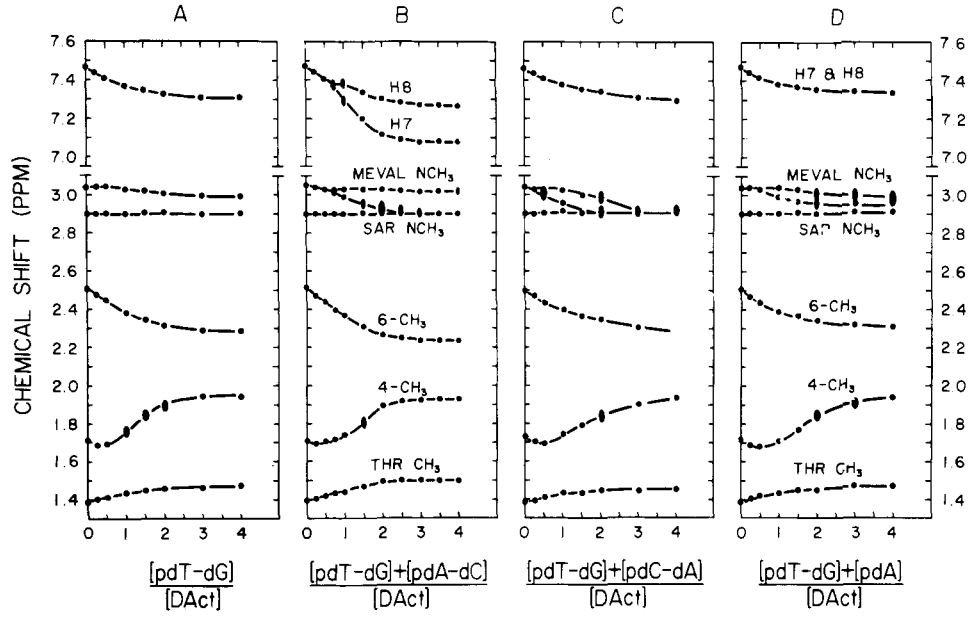


FIGURE 7: Chemical shifts of selected actinomycin D groups plotted as a function of the (total nucleotide)/drug ratio for: (A) pdT-dG; (B) an equimolar mixture of pdT-dG and pdA-dC; (C) an equimolar mixture of pdT-dG and pdC-dA; (D) an equimolar mixture of pdT-dG and pdA. The spectra were recorded at 6 °C, 5 mM actinomycin D.

to this question. However, the titration of actinomycin D with the noncomplementary mixture of pdG-dT + pdC-dA (Figure 4C) does provide some insight into this question. In this titration the H(7) and H(8) resonance remains a singlet until the dinucleotide to actinomycin D ratio is >2:1, and even then the chemical shifts of the H(7) and H(8) protons do not result in a well-resolved AB pattern (Figure 5 in supplementary material). The limiting chemical shift of the 4-$CH_3$ resonance in the pdG-dT + pdC-dA titration is identical with that observed in the pdG-dT + pdA-dC titration (Figures 4C and 4A, respectively), which suggests that the geometry of the complex in the vicinity of the 4-binding site is the same for both of the complexes. On the other hand, the limiting chemical shift of the 6-$CH_3$ resonance at the end of the titration with the mixture of the complementary dinucleotides pdG-dT + pdA-dC is 6 Hz upfield of the limiting chemical shift of the 6-$CH_3$ resonance at the end of the pdG-dT + pdC-dA titration. The two methylvaline $NCH_3$ resonances also show independent titrations. These observations suggest that the predominant form of the actinomycin D complex with the mixture of deoxydinucleotides pdG-dT + pdC-dA is the one shown in Figure 6C.

The $^1H$ chemical shifts for the titration of actinomycin D with a mixture of pdG-dT + pdA (i.e., 5′-dAMP) are shown in Figure 4D. The chemical shifts of the 4-$CH_3$, 6-$CH_3$, and H(7) and H(8) resonances are very similar to those observed in the pdG-dT + pdC-dA titration which leads us to propose that the predominant complex formed is the one in which the pdG-dT is bound in the 4-binding site while dAMP binds in the 6-binding site as schematically illustrated in Figure 6D. Note that in comparing Figures 6C and 6D the only differences in the structures is that the extra cytosine base in the pdG-dT + pdC-dA titration would be expected to provide an additional shielding of 5–10 Hz to *one* of the methylvaline $NCH_3$ resonances (e.g., see Krugh and Neely, 1973b, for a discussion). This is experimentally observed (Figures 4C and 4D), which supports the interpretation of the data in terms of the formation of the complexes shown in Figure 6.

The $^1H$ chemical shifts for a titration of actinomycin D with the mixture pdG-dT + pdA-dA are shown in Figure 4E. The observation of the AB pattern for the H(7) and H(8) resonances clearly indicates the formation of a complex in which one of the deoxydinucleotides is wrapped around the benzenoid portion of the phenoxazone ring. In light of the data in Figures 4C and 4D, we had anticipated that the predominant complex would be one in which the pdA-dA was bound in the 6-binding site in a manner similar to pdC-dA. We therefore expected that one of the methylvaline $NCH_3$ groups would be shifted upfield ~40 Hz as a result of the ring current of the second adenine base (e.g., see Figure 3C and Figure 7 of Krugh and Neely, 1973b). Since we do not observe the large upfield shift of the methylvaline $NCH_3$ resonance and we do observe that the H(7) and H(8) resonance is partially split into an AB pattern, we conclude that one of the dinucleotides is wrapped around the benzenoid portion of the phenoxazone ring.

*Actinomycin D Titrations with Binary Mixtures Involving pdT-dG.* The $^1H$ chemical shift data for the titrations of actinomycin D with pdT-dG and binary mixtures with pdT-dG are shown in Figure 7. The chemical shifts in the pdT-dG titration are consistent with the formation of a complex in which two pdT-dG dinucleotides are bound to the phenoxazone ring of actinomycin D to form a stacked complex, as schematically illustrated in Figure 8A. Note, for example, that the H(7) and H(8) resonance remains a singlet throughout the titration, and that the two methylvaline $NCH_3$ resonances remain equivalent throughout the titration (see also Krugh and Neely, 1973b).

The $^1H$ chemical shifts of the actinomycin D resonances in the titration of actinomycin D with the mixture of pdT-dG + pdA-dC are shown in Figure 7B. The H(7) and H(8) resonances are split into an AB pattern in this titration even though these resonances remain a singlet throughout the titrations of actinomycin D with the individual deoxydinucleotides pdT-dG or pdA-dC. The methylvaline $NCH_3$ resonances titrate individually; one of the methylvaline $NCH_3$ resonances moves in a manner similar to that observed in the pdT-dG titration, while the other methylvaline $NCH_3$ resonance titrates in a manner similar to that observed for the pdG-dT + pdA-dC titration (Figure 4A). The limiting chemical shift of the 4-$CH_3$

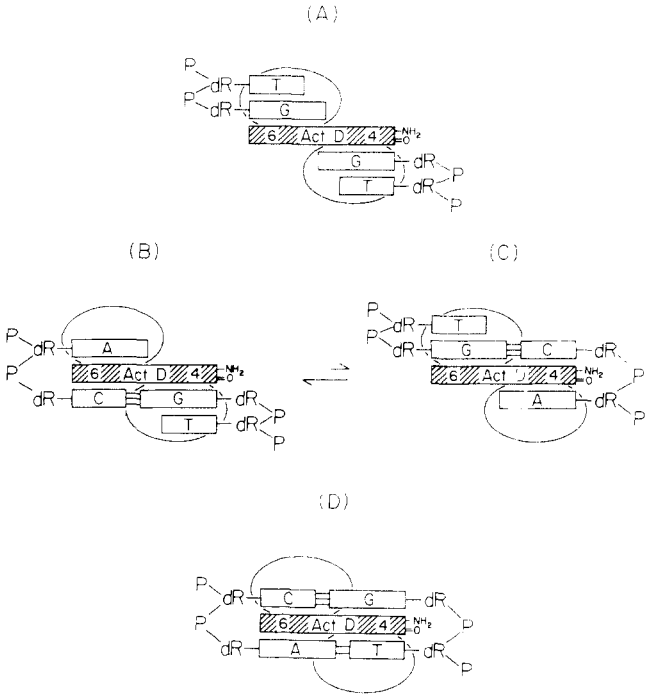


FIGURE 8: Schematic illustrations of actinomycin D complexes: (A) Act D-2 (pdT-dG) complex; (B) Act D-pdT-dG-pdA-dC complex in which pdA-dC is wrapped around the 6-binding site while pdT-dG binds to the 4-binding site; (C) Act D-pdT-dG-pdA-dC complex in which pdT-dG binds to the 6-binding site while pdA-dC is bound to the 4-binding site; (D) a possible intercalated type complex of actinomycin D with the mixture pdT-dG + pdC-dA, which is not formed.

resonance in the pdT-dG + pdA-dC titration is the same as in the pdT-dG titration. On the other hand, the limiting chemical shift of the 6-$CH_3$ resonance in the pdT-dG + pdA-dC titration is 5 Hz more upfield than in the pdT-dG titration and corresponds to the same value observed in the titration of the complementary nucleotides pdG-dT + pdA-dC. All of this data are consistent with the formation of the complex illustrated in Figure 8B in which the pdA-dC is wrapped around the benzenoid portion of the phenoxazone ring while pdT-dG is bound in the 4-binding site. Of course there will be some fraction of the actinomycin D molecules existing in the type of complex illustrated in Figure 8C. However, the chemical shift data indicate that no more than 20% of the actinomycin D molecules exist in this or the other possible alternative complexes.

The $^1H$ chemical shift data for an actinomycin D titration with pdT-dG + pdC-dA are shown in Figure 7C. Although these are complementary dinucleotides, the observation that the H(7) and H(8) resonance remains a singlet clearly indicates that the actinomycin D *does not form* the type of intercalated complex schematically illustrated in Figure 8D. A comparison of the titration behavior of the methylvaline $NCH_3$ groups in the pdT-dG + pdC-dA titration with those observed in the titrations with the individual dinucleotides (Figures 7C, 7A, and 3A, respectively) as well as the chemical shift data for the 4-$CH_3$ and 6-$CH_3$ groups indicate that both pdC-dA and pdT-dG compete for the nucleotide binding sites of actinomycin D. These conclusions are consistent with our previous studies (Krugh and Neely, 1973a,b; Krugh and Chen, 1975).

The $^1H$ chemical shift data for the titration of actinomycin D with the mixture pdT-dG + pdA (i.e., 5′-dAMP) are shown in Figure 7D. The H(7) and H(8) resonance remains a singlet throughout this titration, as expected. The methylvaline $NCH_3$ groups titrate individually, with one of the MeVal $NCH_3$ resonances titrating essentially the same as in the pdT-dG titration (Figure 7A). This is consistent with our previous data (Krugh and Neely, 1973a; Krugh and Chen, 1975) which indicated that dAMP effectively competes with dGMP for the 6-binding site, whereas dGMP binds much stronger than dAMP in the 4-binding site.

*Other Titrations.* The proton chemical shift data for the titration of actinomycin D with the mixture pdG + pdA-dC are shown in Figure 9 (in supplementary material). The appearance of the AB pattern for the H(7) and H(8) proton resonances and the appearance of two individual methylvaline $NCH_3$ resonances indicate that the major complex formed is one in which the pdA-dC is wrapped around the benzenoid portion of the phenoxazone ring of actinomycin D, while the pdG is binding in the 4-binding site. On the other hand, the splitting of the H(7) and H(8) resonances in this titration is less than that observed in the pdT-dG + pdA-dC titration (Figure 7B) which suggests that either pdG competes for the 6-binding site more effectively with pdA-dC than does pdT-dG, or that the conformation of the phosphodiester linkage of pdA-dC which is wrapped around the benzenoid portion of the chromophore is slightly different in the two complexes.

The proton chemical shifts for the titration of actinomycin D with the mixture pdA-dG + pdA-dC are also shown in Figure 9 (in supplementary material). The H(7) and H(8) resonance splits into an AB pattern, as expected. The methylvaline $NCH_3$ resonances move significantly further upfield in this titration than in the other titrations because of the large ring currents of the adenine base.

*Actinomycin D Titrations with a Mixture of pdG-dG + pdC-dC.* The chemical shifts of the actinomycin D protons and nucleotide protons are shown as a function of the nucleotide to drug ratio for titrations of actinomyin D with pdG-dG, pdC-dC, and the mixture of pdG-dG + pdC-dC in Figure 10. The magnitude of the cytidine ring current is smaller than the ring current of guanine. Since actinomycin D is partially dimerized at the start of the titration, we would expect to see downfield shifts of the actinomycin D protons in the pdC-dC titration if a complex is formed. The invariance of the chemical shifts of the actinomycin D and the pdC-dC protons (Figure 10C) indicates that pdC-dC does not form a complex with actinomycin D under the present experimental conditions. In the actinomycin D titration with pdG-dG (Figure 10A), the behavior of the 4-$CH_3$, the 6-$CH_3$, and the H(7) and H(8) resonances appears similar to that observed in the titration with 5′-dGMP. Note that in the pdG-dG titration the methylvaline $NCH_3$ resonances move upfield ~0.4 ppm. This large upfield shift of the methylvaline $NCH_3$ resonances is similar to that observed in the titration of actinomycin D with pdA-dG and, as previously discussed (Krugh and Neely, 1973b), this indicates that a purine ring is in the vicinity of the methylvaline $NCH_3$ group. The H(7) and H(8) resonance remains as a singlet throughout the titration, although there is a noticeable broadening of this resonance between nucleotide to drug ratios of 0.3 to 1.5. We thus conclude that the predominant complex formed is the one in which two pdG-dG molecules bind to the phenoxazone ring to form a stacked complex (Figure 11A). The sandwich type of complex (Figure 11B) is undoubtedly in equilibrium with the stacked complex, especially during the early stages of the titration where the H(7) and H(8) resonance is slightly broadened, but the limiting chemical shifts and the observation that the H(7) and H(8) resonance is a singlet strongly support the predominance of the stacked complex

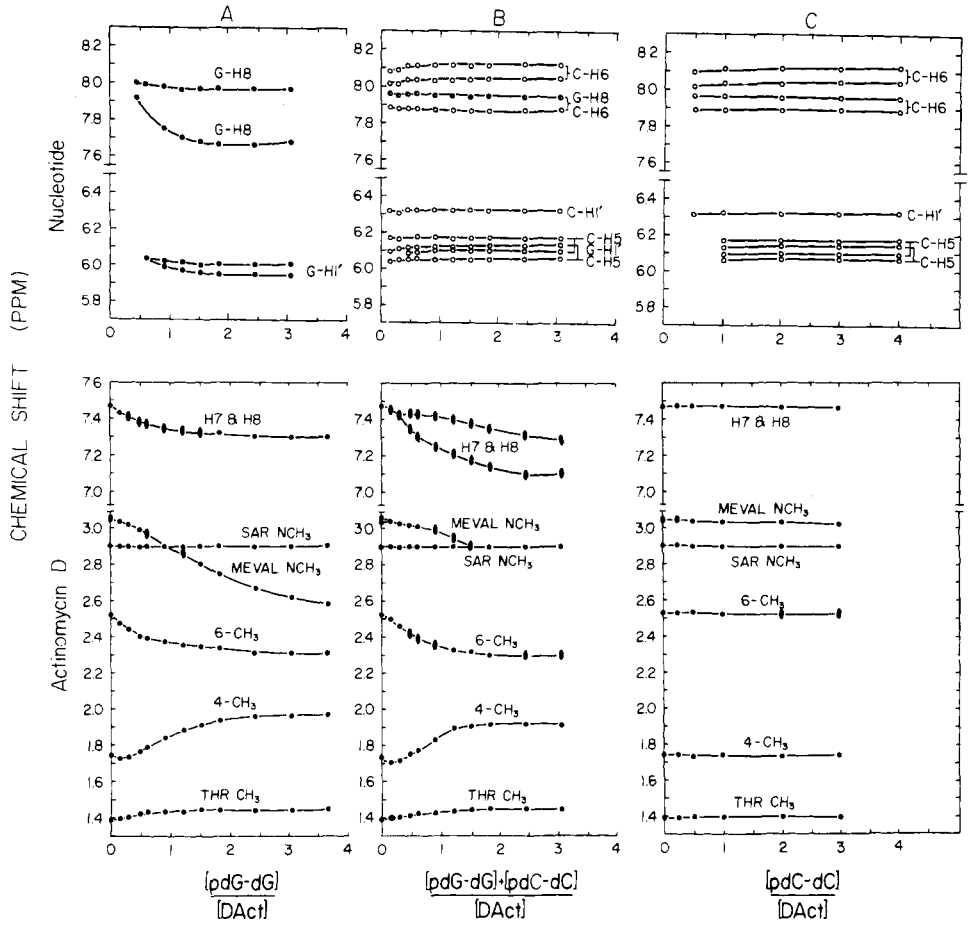


FIGURE 10: Chemical shifts of selected actinomycin D and nucleotide resonances plotted as a function of the (total nucleotide)/drug ratio for: (A) pdG-dG; (B) an equimolar mixture of pdG-dG and pdC-dC; (C) pdC-dC. The spectra were recorded at 6 °C, 5 mM actinomycin D.

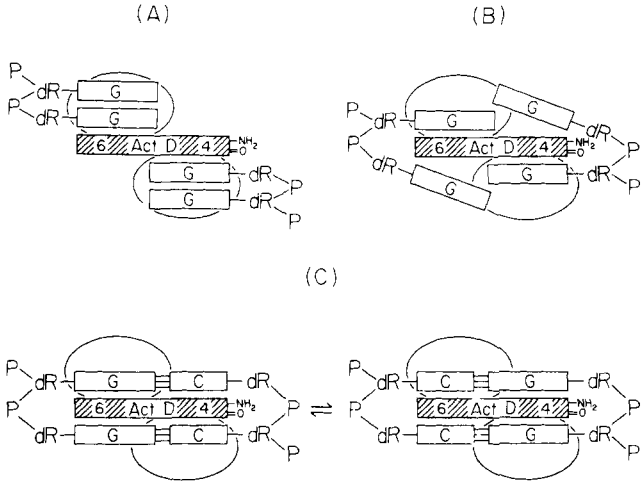


FIGURE 11: Schematic illustrations of actinomycin D complexes. (A) A stacked complex for Act D·2 (pdG-dG); (B) a sandwich type complex formation for Act D·2(pdG-dG); (C) two different conformations for an intercalated type complex of Act D·pdG-dG·pdC-dC.

(Figure 11A) for [pdG-dG]/[Act D] ratios ≥ 2:1. For example, the limiting chemical shifts in a titration of actinomycin D with 5′-dGMP (Krugh and Chen, 1975) were 7.30 ppm (H(7) and H(8)), 2.32 ppm (6-$CH_3$), and 2.00 ppm (4-$CH_3$) which compares very well with the present data for the pdG-dG titration where the limiting shifts were 7.30 ppm (H(7) and H(8)), 2.31 ppm (6-$CH_3$), and 1.97 ppm (4-$CH_3$).

In the titration of actinomycin D with the complementary mixture of dinucleotides pdG-dG + pdC-dC we observe that the H(7) and H(8) resonance is split into an AB pattern which is indicative of the formation of an intercalated complex (Figure 11C). In addition, the methylvaline $NCH_3$ resonances move upfield only 0.15 ppm which also indicates that the stacked complex (Figure 11A) is no longer the predominant complex. From the values of the limiting chemical shifts of the 4-$CH_3$ and the 6-$CH_3$ resonances we conclude that the intercalated complex consists of an approximately 50:50 mixture of the two complexes schematically illustrated in Figure 11C. If one of these two complexes were in great excess, then either the 4-$CH_3$ or the 6-$CH_3$ resonance would experience the large magnetic shielding of the two guanine bases, while the other resonance would experience only the small shielding of the two cytosine rings.

The chemical shifts of the nucleotide resonances were also recorded (Figure 10). One of the G-H(8) resonances moves upfield ~30 Hz during the titration of actinomycin D with pdG-dG. In the titration of actinomycin D with the mixture of pdG-dG + pdC-dC this G-H(8) resonance does not shift upfield, which clearly shows that the presence of pdC-dC influences the geometry of the actinomycin D complex with pdG-dG. The C-H(5) and C-H(6) resonances of pdC-dC are not significantly different in either the pdC-dC or pdG-dG + pdC-dC titrations with actinomycin D. Thus the geometry of the pdG-dG + pdC-dC complex must be one in which the C-H(5) and C-H(6) protons experience only a negligible ring current from the phenoxazone ring of actinomycin D. Using

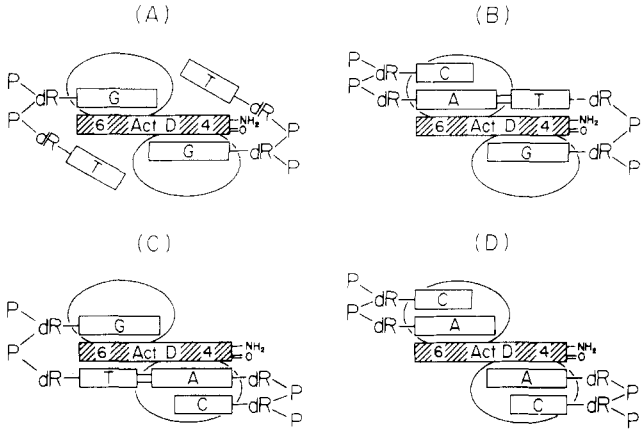


FIGURE 12: Schematic illustrations of four possible actinomycin D complexes in the Act D·pdG-dT·pdC-dA solutions. (A) A sandwich type complex for the actinomycin D·2(pdG-dT) complex; (B) Act D·pdG-dT·pdC-dA complex in which pdG-dT is wrapped around the 4-binding site and base paired to the pdC-dA stacked in the 6-binding site; (C) Act D·pdG-dT·pdC-dA complex in which pdG-dT is bound in the 6-binding site and pdC-dA is bound in the 4-binding site; (D) Act D·2(pdC-dA) complex.

Patel's (1974) approximate ring current contours for the phenoxazone ring and the general features of the intercalated complex proposed by Sobell and Jain (1972), we predict that the C-H(5) and C-H(6) protons would experience an average ring current shift of less than 0.05 ppm for the intercalated complexes shown in Figure 11C. Thus the chemical shift data for the nucleotide resonances are consistent with the formation of an intercalated complex in the pdG-dG + pdC-dC titration.

## Discussion

In the titrations of actinomycin D with the mixtures of nucleotides, the interpretation of the changes in the chemical shifts is complicated by the simultaneous multiple equilibria present in these systems. As a result, we have concentrated our attention on the spectra in which the nucleotide/drug ratios are 2:1 or larger, because these spectra generally approximate the condition in which both of the nucleotide binding sites of actinomycin D have bound one of the nucleotides. The dimerization of actinomycin D, which influences the chemical shifts of the actinomycin D resonances during the early stages of the titration, is not important in the spectra for nucleotide to drug ratios $\geq$2:1. However, there are still four possible complexes for the 2:1 nucleotide–actinomycin D complexes, as schematically illustrated in Figure 12 for the titration of actinomycin D with the pdG-dT + pdC-dA mixture. The changes in the infinite dilution chemical shifts of the actinomycin D resonances that result from the formation of a complex with the nucleotides (i.e., the complexation shifts) may be used to deduce information on the geometry of the complexes, but the presence of four possible complexes makes it difficult to determine which form of the complexes is predominant. However, the behavior of the H(7) and H(8) resonances, as well as the other actinomycin D resonances, provides insights into the type of complexes formed. For example, a comparison of the H(7) and H(8) resonances in the titration of actinomycin D with the complementary mixture pdG-dT + pdA-dC (Figure 4A) and the titration with the mixture pdG-dT + pdC-dA (Figure 4C) shows that in the latter titration at nucleotide to drug ratios <2:1 the nucleotide bound in the 6-binding site does not wrap around the benzenoid portion of the phenoxazone ring. This eliminates complexes 12A and 12C (Figures 12A and 12C, respectively) from being the major complexes. The observation of two different titration curves for the MeVal $NCH_3$ resonances indicates that two different nucleotides are bound to actinomycin D. We thus conclude that complex 12B is the predominant complex formed at a nucleotide to drug ratio of 2:1. As excess nucleotides are added, we expect that pdG-dT will compete with pdC-dA for the 6-binding site; this is consistent with the observation of the AB pattern for the H(7) and H(8) resonances at nucleotide to drug ratios $\geq$ 2:1 (Figures 4C and 5 (Figure 5 in supplementary material)). These data suggest that the adenine nucleotide binds stronger to the 6-binding site than to the 4-binding site, while the guanine nucleotide binds to both the 4-binding site and the 6-binding site. We do not feel confident in trying to extract relative binding constants from either the chemical shifts or the analysis of the H(7) and H(8) resonances because of the uncertainties involved. The paramagnetic-induced relaxation experiments presented in the following paper (Chiao and Krugh, 1977) support the present data and provide more quantitative information.

The appearance of the well-resolved AB pattern for the H(7) and H(8) protons in the titration of actinomycin D with the pdT-dG + pdA-dC mixture (Figure 7B) provides an excellent example of the preferential binding because the H(7) and H(8) resonance remains a singlet in the titrations of actinomycin D with the individual dinucleotides (Figures 7A and 3B, respectively). This almost certainly means that complex 8B predominates in aqueous solution for nucleotide/drug ratios $\geq$ 2:1. We estimate that >70% of the actinomycin D molecules are in this type of complex. The titration of actinomycin D with the pdT-dG + pdC-dA mixture confirms these conclusions. In this titration the observation of a singlet for the H(7) and H(8) resonance indicates that the nucleotide bound in the 6-binding site does not wrap around the phenoxazone ring. A comparison of these two titrations in which the two sequence isomers pdA-dC and pdC-dA are used in combination with pdT-dG provides a clear demonstration of the unique ability of actinomycin D to preferentially bind the deoxydinucleotides.

The titrations of actinomycin D with pdG-dG, pdC-dC, and the equimolar mixture of these complementary deoxydinucleotides illustrate the role of complementarity and the influence of Watson–Crick hydrogen bond formation on the geometry of the complexes. The chemical shifts of the actinomycin D resonances in the pdG-dG titration are consistent with the formation of a stacked complex (Figure 11A), which is analogous to the other pdN-dG nucleotides (Krugh and Neely, 1973b). In the pdC-dC titration the pyrimidine bases in pdC-dC show no evidence of association with actinomycin D. However, in the titration with the equimolar mixture of the complementary dinucleotides pdG-dG + PDC-dC, the analysis of the chemical shifts shows that the predominant complex is the one in which actinomycin D has formed a miniature intercalated complex (which serves as a model for the binding of actinomycin D to a (dG-dG)·(dC-dC) sequence on DNA). The two possible complexes (Figure 11C) exist in approximately equal amounts. We thus conclude that the free energy change for the binding of pdG-dG to the 4- and 6-binding sites is about the same. This is not surprising because dGMP binds about equally to the two binding sites. At the present time it is not possible to unequivocally determine the relative proportions of the two possible intercalated complexes in the pdG-dT + pdA-dC titration (Figure 6B). The binding preferences exhibited in the other mixtures of dinucleotides suggest

that the predominant complex should be the one in which the guanine is bound in the 4-binding site, while the adenine is bound in the 6-binding site (i.e., the complex on the right in Figure 6B). A more detailed discussion will be presented in the following paper (Chiao and Krugh, 1977).

Supplementary Material Available

Figure 2, 5, and 9 (4 pages). Ordering information is given on any current masthead page.

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# Actinomycin D Complexes with Oligonucleotides as Models for the Binding of the Drug to DNA. Paramagnetic Induced Relaxation Experiments on Drug–Nucleic Acid Complexes†

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ABSTRACT: Mn(II) ions have been used as a paramagnetic probe to investigate the geometry of drug-oligonucleotide complexes. Nuclear magnetic resonance and electron spin resonance experiments show that Mn(II) ions bind approximately two orders of magnitude stronger to the 5′-terminal phosphate group than to the 3′-5′ phosphodiester linkage of deoxydinucleotides. By using mixtures of nucleotides in which only one nucleotide contains a terminal phosphate group, the location of the Mn(II) ion in the drug-nucleotide-Mn(II) complexes may be preselected. The paramagnetic induced relaxation of the nuclear spin systems in these complexes has been used to investigate the geometry of these complexes. These data confirm that actinomycin D is able to recognize and preferentially bind guanine (as opposed to adenine) nucleotides in the quinoid portion of the phenoxazone ring, while both adenine and guanine will bind to the benzenoid portion of the phenoxazone ring. These results suggest that stacking forces are primarily responsible for the general requirement of a guanine base when actinomycin D binds to DNA.

In the preceding manuscript (Krugh et al., 1977) we have investigated the geometry of the complexes formed between actinomycin D and mixtures of deoxymono- and dinucleotides by analyzing the changes in the proton chemical shifts of the actinomycin D and nucleotide resonances. In the present manuscript we show that the paramagnetic Mn(II) ion binds much stronger to terminal phosphate groups than to phosphodiester groups, and we utilize this phenomenon to specifically locate the paramagnetic probe in actinomycin D-nucleotide-manganese(II) complexes. Paramagnetic metal ions have been previously used as a probe to obtain structural information in a variety of biological systems. Electron spin resonance (ESR)[1] may be used to directly monitor changes in the environment (e.g., ligation) of the paramagnetic metal ion,

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[1] Abbreviations used: NMR, nuclear magnetic resonance; ESR, electron spin resonance.