Actinomycin D–Mononucleotide Interactions as Studied by Proton Magnetic Resonance†

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ABSTRACT: The 100-MHz proton magnetic resonance spectra of actinomycin D complexed with deoxyguanosine 5′-monophosphate, guanosine 5′-monophosphate, deoxyadenosine 5′-monophosphate, and adenosine 5′-monophosphate are presented and discussed. The spectra were recorded for various nucleotide to drug ratios in order to determine the stoichiometry of the complexes. The results clearly show that actinomycin D has two binding sites for deoxyguanosine 5′-monophosphate and guanosine 5′-monophosphate. The adenine nucleotides also bind to the same binding sites. The geometries of the complexes were determined from an analysis of the induced chemical shifts of the actinomycin D protons upon complex formation. The results show that the complex consists of stacking one of the guanine bases on each side of the actinoymcin D chromophore. The geometry of the actinomycin D–deoxyguanosine 5′-monophosphate complex is similar to that observed in the solid state complex of actinomycin D and deoxyguanosine (Sobell, H. M., Jain, S. C., Sakore, T. D., and Nordman, C. E. (1971), *Nature (London) 231*, 200). The other nucleotides also formed complexes with similar geometries. The incremental addition of nucleotides has provided information on the relative magnitudes of the binding constants. Deoxyguanosine 5′-monophosphate and guanosine 5′-monophosphate appear to have approximately equal binding constants for the binding site on the benzenoid portion of the chromophore. However, the deoxyguanosine 5′-monophosphate appears to have a much larger binding constant than guanosine 5′-monophosphate for the binding site on the quinoid portion of the chromophore.

Actinomycin D (Figure 1) is an important antibiotic that binds to DNA and inhibits RNA synthesis (Kirk, 1960; Kersten *et al.*, 1960). The interaction of actinomycin D and DNA has been widely studied by a number of techniques (for reviews see Reich and Goldberg, 1964; Sobell, 1972). Binding of the drug to DNA may be monitored by observing the change in the visible spectrum of actinomycin D as it binds to DNA. These changes may be mimicked by complexing actinomycin D with a variety of mononucleosides and nucleotides (Kersten, 1961; Reich, 1964) but the apparent association constant is three orders of magnitude lower (10^3 *vs.* 10^6) than the association constant for the binding of the drug to DNA. Sobell and coworkers (1971) have recently determined the structure of a crystalline complex of actinomycin D and deoxyguanosine and have proposed a model for the binding of the drug to DNA (Sobell, 1972; Sobell and Jain, 1972). This model predicts that actinomycin D will intercalate into a dG-dC sequence on DNA with the cyclic pentapeptide rings located in the minor groove. The intercalation model had been proposed earlier by Müller and Crothers (1968) and supported by Waring (1970) and Wang (1971). We recently investigated the interaction of actinomycin D and a series of deoxydinucleotides (Krugh, 1972) which showed that actinomycin D has a preference for the dG-dC sequence of DNA. This work also clearly demonstrated the stereochemical specificity of the interaction of actinomycin D and the deoxydinucleotides. The results of a proton magnetic resonance (pmr) investigation of the complex formation of actinomycin D and mononucleotides are reported in this paper. We have found that actinomycin D forms a complex with two deoxyguanosine 5′-monophosphate molecules in aqueous solution and that the chemical-shift changes induced in the actinomycin D resonances are consistent with the complex having a geometry similar to that observed for a deoxyguanosine–actinomycin D complex in the solid state (Sobell *et al.*, 1971). A following paper (Krugh and Neely, 1973) discusses the pmr investigation of the complexes of actinomycin D and the deoxydinucleotides.

The actinomycin D–dGMP[1] complex has been studied by pmr before, in both 1:1 and 1:2 mixtures (Danyluk and Victor, 1970; Arison and Hoogsteen, 1970). Both previous studies also led to the conclusion that the complex formation involves a stacking of the aromatic ring systems. The present experiments demonstrate the importance of monitoring the actinomycin D spectrum as the nucleotide is incrementally added to the solution. The additional information gained by this technique is well worth the additional experimental time required. The complete assignment of the pmr spectrum of actinomycin D in D_2O has been reported previously (Arison and Hoogsteen, 1970; Angerman *et al.*, 1972) and these assignments were used in the present study. Actinomycin D exists predominantly in the dimer form (Crothers *et al.*, 1968) at concentrations $>10^{-4}$. Angerman *et al.* (1972) have recently reported an excellent pmr study of the aggregation of actinomycin D in D_2O and have shown that the dimer results from a stacking of the actinocyl groups (phenoxazone rings) with one chromophore inverted with respect to the second. The formation of dimers is an important consideration in a detailed analysis of the actinomycin D–nucleotide spectra. We have independently studied the effect of dimerization on the chemical shifts of actinomycin D under appropriate experimental conditions.

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[1] The abbreviations used are: dGMP, deoxyguanosine 5′-monophosphate; GMP, guanosine 5′-monophosphate; dCMP, deoxycytidine 5′-monophosphate; AMP, adenosine 5′-monophosphate.

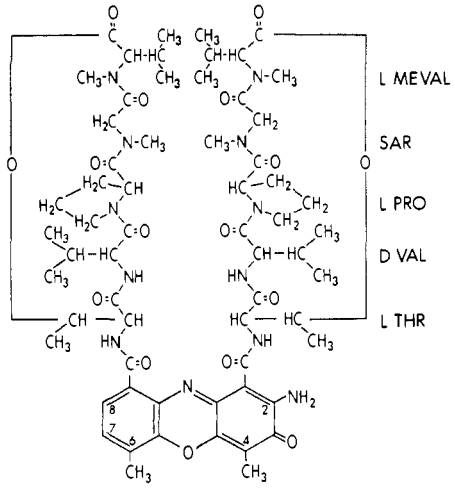

FIGURE 1: Structural formula of actinomycin D. Abbreviations used are: Thr = threonine; Val = valine; Pro = proline; Sar = sarcosine; MeVal = methylvaline.

Experimental Section

Actinomycin D was a gift of Merck Sharpe and Dohme and was used without further purification except that a volatile impurity was removed by placing the actinomycin D under vacuum for 1 hr immediately prior to use. The mononucleotides were purchased from Sigma Chemical Co. and used without purification. All samples were dissolved in a 5 mM potassium phosphate buffer in D_2O and the pH was adjusted with NaOD and DCl until the pH 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 ϵ_{425} 23,500 (Smith, 1963). The concentrations of the mononucleotide solutions were also measured spectrophotometrically.

The actinomycin D–nucleotide complexes were investigated by first measuring the pmr spectra of the stock actinomycin D solution. A small aliquot of a concentrated nucleotide solution was then added and the spectrum was rerun. This procedure was repeated a number of times until the ratio of [nucleotide]/[actinomycin D] was >3. The original actinomycin D solution was diluted by no more than 30% during the titrations. The range of concentrations was from 0.02 to about 0.015 M. The actinomycin D spectrum is relatively unchanged in this concentration range (Figure 2), so the observed changes in the chemical shifts of the actinomycin D protons are a result of the interactions of the nucleotides and the drug.

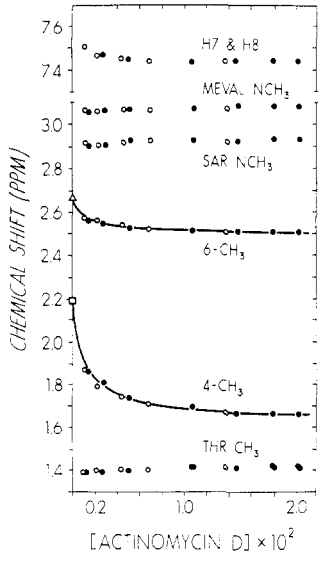

FIGURE 2: Concentration dependence of important groups of actinomycin D in D_2O at 3°.

Nuclear magnetic resonance (nmr) experiments were performed on a JEOL 4H-100 spectrometer. A Model JMN-RA-1 spectrum accumulator was used for time averaging. The temperature was regulated at 3 ± 1° by a Model VES-VT-3 temperature controller. The temperature was determined in the usual manner using a methanol sample. Cylindrical microcells (Wilmad Glass Co.) of approximately 175-μl volume were used for all experiments. 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 start of each experiment). We did not observe any influence of the reference compound on the pmr spectrum of actinomycin D or the nucleotides.

Results

The chemical shifts of selected actinomycin D protons are shown as a function of concentration in Figure 2. The solid circles represent actinomycin D diluted with the 5 mM phosphate buffer. The open circles correspond to an actinomycin D sample which was diluted with 5 mM phosphate buffer containing NH_4Cl of ionic strength approximately equal to that of the nucleotide solutions used in these experiments. The results of the two studies are not significantly different and are in excellent agreement with the recent results of Angerman *et al.* (1972). The association of actinomycin D can be well represented by a simple dimerization reaction

$$2A \rightleftharpoons A_2 \qquad (1)$$

with $K_d = [A_2]/[A]^2$ M^{-1}. The concentration dependence of the chemical shifts can be used to determine the equilibrium constant and the chemical shifts of the actinomycin D protons in the monomeric and dimeric forms. The procedure is outlined in detail by Angerman *et al.* (1972) and will not be repeated here. We selected the value of the equilibrium constant and used a noninear least-squares regression analysis program to calculate δ^0_A, the limiting shift of the actinocyl protons in the monomer, and $\delta^0_{A_2}$, the limiting shift of the actinocyl protons in the dimer. Table I lists the values which produced the best fit of the calculated curve to the experimental data for the dilution of actinomycin D with buffer solution (solid circles in Figure 2). A 25% error in the value of K_d would change the calculated values of the limiting shifts by less than the standard deviations listed in Table I. The present ionic strength is intermediate between the two cases studied by Angerman *et al.* (1972) and, as expected, the values given in Table I fall between the values reported earlier. Our results are in agreement with those of Angerman *et al.* and support the conclusion that the dimer results from a vertical stacking of the actinocyl groups with one chromophore preferentially inverted with respect to the other.

Actinomycin D–Mononucleotide Interactions. The composite spectra for the interaction of actinomycin D with 5′-dGMP and 5′-GMP are shown in Figure 3. The chemical shifts for

TABLE I: Chemical-Shift Parameters Determined from a Nonlinear Least-Squares Analysis of the Dilution Data of Actinomycin D at 3 ± 1°.[a]

	δ_A^0 (ppm)	$\delta_{A_2}^0$ (ppm)
4-CH_3	2.20 ± 0.05	1.56 ± 0.01
6-CH_3	2.67 ± 0.01	2.476 ± 0.005
H7 & H8	7.60 ± 0.02	7.453 ± 0.005

[a] The values given are for the dilution of actinomycin D with a buffer solution using $K_d = 2.9 \times 10^3$ M^{-1}. The stated uncertainties are standard deviations based upon this value of K_d.

the interaction of actinomycin D with dGMP and GMP are plotted in Figure 4. In Figure 4 the chemical shifts of the actinomycin D protons cease changing when the ratio [dGMP]/[actinomycin D] reaches a value of 2. The invariance of the chemical shifts when the ratio [dGMP]/[actinomycin D] > 2 and the shape of the titration curves provide solid evidence that actinomycin D forms a complex with *two* dGMP molecules. It should also be noted that the two sarcosine *N*-methyl groups, the two methylvaline *N*-methyl groups, and the two threonine methyl groups are equivalent throughout the titration with dGMP; this indicates that the binding sites on actinomycin D have about equal binding constants for the association of dGMP. The formation of 2:1 complexes was also observed for the deoxydinucleotide complexes with actinomycin D (Krugh and Neely, 1973). These results are also consistent with the fact that actinomycin D cocrystallized with *two* deoxyguanosine molecules (Sobell *et al.*, 1971). The resonance of the 4-methyl group initially moves upfield and then rapidly moves downfield as the [dGMP]/[actinomycin D] ratio is increased. The observed changes in the chemical shifts of the actinomycin D protons arise from two sources: (1) the disruption of the actinomycin D dimers as a result of complex formation with the nucleotides and (2) the change in the chemical shifts that are a direct result of complex formation with the nucleotides. These induced chemical shifts are a function of the geometry of the complex. We are interested in the net chemical-shift changes for the complexation of the actinomycin D monomer with two dGMP molecules. The value of the induced shifts may be calculated from the chemical shifts of the actinomycin D–$(dGMP)_2$ complex, and the infinite dilution shifts (*i.e.*, the monomer chemical shifts) of actinomycin D, δ_A^0. These values for the 4-CH_3 group (220–200 Hz) show that the 4-CH_3 group has a net *upfield* shift of 20 Hz in going from the actinomycin D monomer to the actinomycin D–$(dGMP)_2$ complex. During the titration the chemical shift of the 4-CH_3 moved *downfield* because the destacking of the dimers produced a downfield shift of 53 Hz while the nucleotides induced an upfield shift of only 20 Hz. The situation is reversed for the 6-CH_3 group where the destacking of the dimers shifts the 6-CH_3 group 17 Hz downfield while the nucleotide binding induces an upfield shift of 34 Hz (267–233). Complex formation with dGMP shifts the H-7 and H-8 protons 32 Hz upfield with respect to the actinomycin D monomer (760–728). Note that the H-7 and H-8 protons remain equivalent throughout the titration with dGMP. All the above shifts are consistent with a vertical base stacking of the nucleotides and the phenoxazone ring of actinomycin D as will be discussed later.

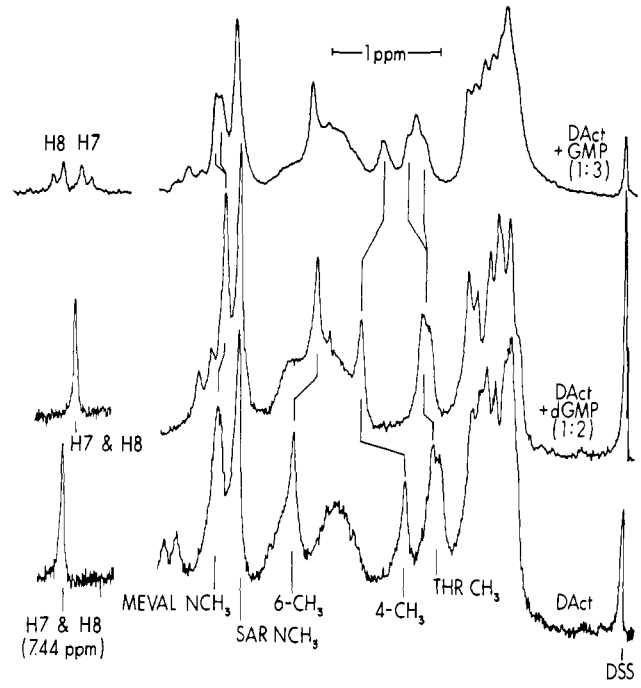

FIGURE 3: 100-MHz proton spectrum of: (a) actinomycin D; (b) actinomycin D and dGMP in a 1:2 ratio; (c) actinomycin D and GMP in a 1:3 ratio.

The titration curves for the interaction of actinomycin D and 5′-GMP are in sharp contrast with those for 5′-dGMP. As the ratio of [GMP]/[actinomycin D] is increased the two threonine methyl groups and the two methylvaline N–CH_3 groups become nonequivalent. The H-7 and H-8 resonances split into a well-resolved AB pattern as shown in Figures 3 and 4. The shape of the 4-CH_3 curve is indicative of the reduced interaction between actinomycin D and GMP observed in the optical experiments (Kersten, 1961). The 6-CH_3 curve is much the same for both dGMP and GMP, an important point that will be discussed later.

The data for a titration of actinomycin D with 5′-TMP are shown in Figure 5. It is clear that TMP does not interfere with the self-association of actinomycin D, which is consistent with previous nmr studies (Danyluk and Victor, 1970) and optical

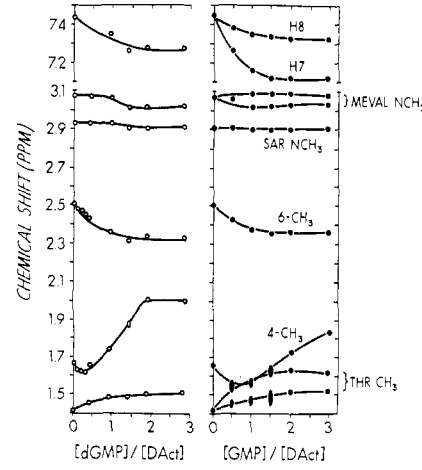

FIGURE 4: Chemical shifts of selected actinomycin D groups plotted as a function of the nucleotide/drug ratio for deoxyguanosine 5′-monophosphate and guanosine 5′-monophosphate.

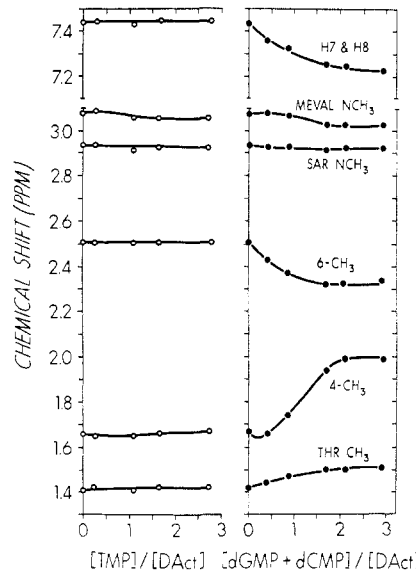

FIGURE 5: Chemical shifts of selected actinomycin D groups plotted as a function of the nucleotide/drug ratio for thymidine 5′-monophosphate and an equimolar mixture of deoxyguanosine 5′-monophosphate and deoxycytidine 5′-monophosphate.

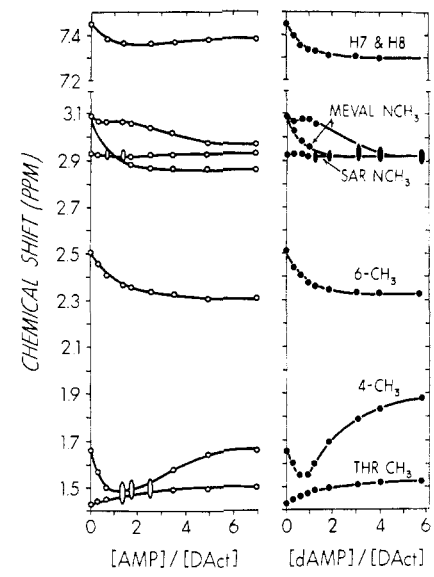

FIGURE 6: Chemical shifts of selected actinomycin D groups plotted as a function of the nucleotide/drug ratio for deoxyadenosine 5′-monophosphate and adenosine 5′-monophosphate.

studies (Kersten, 1961). The data for the interaction of actinomycin D and an equimolar mixture of 5′-dGMP and 5′-dCMP are also shown in Figure 5. These curves are practically indistinguishable from the curves for dGMP alone, which is consistent with the previous optical experiments (Krugh, 1972), and indicates that the presence of dCMP does not drastically affect the binding of dGMP. The chemical shifts of the nucleotide protons were also monitored and suggest that the dCMP does associate with the actinomycin D–dGMP complex (Neely, J. W., and Krugh, T. R., 1972, unpublished results). These results are not conclusive and experiments are currently in progress to investigate this interesting question.

Actinomycin D will also complex with 5′-dAMP and 5′-AMP. The data for these titrations are shown in Figure 6. The most pronounced difference between these two sets of curves appears in the MeVal N–CH_3 curves and the 4-CH_3 curves. Actinomycin D has two MeVal N–CH_3 groups that are magnetically equivalent at the beginning of the titrations. As the adenine nucleotides are added the MeVal N–CH_3 groups begin to exhibit different chemical shifts. Note that the two MeVal N–CH_3 groups become approximately equivalent at the end of the titration with dAMP, while these two signals are separated by $\sim$10 Hz at the end of the AMP titration. The net upfield shielding of the 4-CH_3 group in the actinomycin D–AMP complex is $\sim$25 Hz larger than the upfield shielding observed in the actinomycin D–dAMP complex. This is clear evidence that the introduction of the 2′-hydroxy group to a dAMP molecule bound in the 4-binding site[2] results in a shift of the geometry of the complex which places the adenine base more squarely over the 4-CH_3 group. This change in the geometry of the complex would allow the 2′-hydroxy group of AMP to form a hydrogen bond with the 3-carbonyl oxygen on the phenoxazone ring. The chemical shifts of the protons associated with the 6-binding site demonstrate that the introduction of a 2′-hydroxy group produces much smaller changes in the molecular geometry of the complex. In going from dAMP to AMP the net upfield shift of the 6-CH_3 group is increased an additional 3–5 Hz while the H-7 and H-8 protons are shifted upfield $\sim$8 Hz less. The 6-CH_3 curve, one of the MeVal N–CH_3 curves, and the H-7 and H-8 curves all have about the same shape, which indicates that each of these curves is monitoring the formation of the complex of actinomycin D and the AMP molecule binding in the 6-binding site. The 4-CH_3 curve and the other MeVal N–CH_3 curve reach their limiting value at about the same ratio of [dAMP]/[actinomycin D] and most likely monitor the association of actinomycin D and the second AMP molecule to form the actinomycin D–$(AMP)_2$ complex. There are most likely only two binding sites for the adenine nucleotides[3] since the chemical shifts of the 6-CH_3 and the H-7 and H-8 protons are invariant above [dAMP]/[actinomycin D] ratios $\geq$1.8.

Discussion

Interaction with 5′-dGMP. The net upfield shifts for the 4-CH_3, 6-CH_3, and H-7 and H-8 resonances upon interaction of actinomycin D with dGMP are consistent with a stacking of the guanine base and the phenoxazone ring. The aromatic character of the bases in the nucleotides is a result of delocalized π electrons. When placed in a magnetic field the induced motion of the π electrons will produce a secondary magnetic field which will affect the chemical shifts of adjacent nuclei. The direction and the magnitude of the induced shift will depend upon the location of the nucleus with respect to the π electrons. Vertical base stacking will lead to upfield shifts of the protons with respect to the isolated molecules. The geometry of the complexes may be qualitatively determined by comparing the calculated upfield shifts (based upon a particular orientation of the rings and the intermolecular shielding values of the nucleotides calculated by Giessner-Prettre and Pulman (1970) with the observed induced upfield shifts. The

[2] In the solid state complex the two guanine rings were located on opposite sides of the phenoxazone ring. We define the two binding sites as the 4-binding site (for the guanine stacked over the quinoid portion of the phenoxazone ring) and the 6-binding site (for the guanine stacked above the benzenoid portion of the phenoxazone ring).

[3] See Added in Proof.

magnitudes of the shifts are consistent with the complex having a geometry similar to the crystalline complex of deoxyguanosine and actinomycin D (Sobell *et al.*, 1971) as shown in Figure 7. In this configuration the two cyclic pentapeptide groups are related by a twofold symmetry axis and both of the dGMP molecules may form identical hydrogen bonds to the peptide rings. The guanine 2-amino group forms a strong hydrogen bond to the carbonyl oxygen of the L-threonine residue, while a weaker hydrogen bond connects the guanine ring nitrogen N-3 with the N–H group on the same L-threonine residue. The sarcosine N–CH_3 groups are on the top of the pentapeptide rings and, as expected, their chemical shifts are not affected by the introduction of the guanine base. The MeVal N–CH_3 groups are above (and below) the phenoxazone ring and experience an upfield shift. However, the magnitude of the upfield shift is not much larger than might be anticipated from the dilution experiments (Figure 2), and thus the orientation of these groups with respect to the bound dGMP molecules is somewhat uncertain.

The net upfield shift of the 4-CH_3 group is 0.20 ppm while the 6-CH_3 group is shifted upfield 0.34 ppm in the actinomycin D–$(dGMP)_2$ complex. These values indicate that the two guanine residues in the tertiary actinomycin D–$(dGMP)_2$ complex have different spatial relationships with respect to the two methyl groups on the chromophore. A consideration of the geometry observed in the solid state complex of actinomycin D and deoxyguanosine (Sobell *et al.*, 1971) shows that a small change in the orientation of a guanine ring (with respect to the phenoxazone ring) could markedly affect the magnitude of the induced upfield shift of the respective methyl group. In fact, the guanine in the 6-binding site in the solid state complex was located a little "closer" to the 6-methyl group than the other guanine was to the 4-methyl group (*e.g.*, see Figure 5 of Jain and Sobell, 1972). The nmr results show that the induced upfield shift of the 6-methyl group (0.34 ± 0.05 ppm) is larger than the induced upfield shift of the 4-methyl group (0.20 ± 0.05 ppm), which is amazingly consistent with the solid state geometry. However, we do not wish to overemphasize this point because the uncertainties in both the theoretical calculations and the experiments allow only qualitative statements about the geometries of the complexes. The previous nmr study of Arison and Hoogsteen (1970) on an actinomycin D–$(dGMP)_2$ complex gave upfield shifts of 0.21 and 0.24 ppm for the 4-CH_3 and 6-CH_3 groups, respectively. Both the present results and the previous results are based upon the determination of the chemical shifts of the actinomycin D monomer. Arison and Hoogsteen used dimethylformamide to solubilize and destack the actinomycin D and they estimated the monomer shifts in pure D_2O by extrapolation of the shifts to 0% dimethylformamide. The marked sensitivity of the actinocyl proton chemical shifts to changing solvent conditions is sufficient to explain the observed differences with the present data. In addition, the previous experiments (Arison and Hoogsteen, 1970) were performed at 32° while the present experiments were performed at 3°, which also may account for the observed differences.

The data in Figure 4 show that the chemical shifts of the actinomycin D protons stop changing when the ratio of [dGMP]/[actinomycin D] equals two, which demonstrates that the actinomycin D–$(dGMP)_2$ complex predominates at this concentration ratio. Each actinomycin D can bind two guanine nucleotides (Sobell *et al.*, 1971; Krugh, 1972) with one nucleotide binding on each side of the chromophore. The two binding sites are not intrinsically identical since the crystalline complex (Sobell *et al.*, 1971) had one guanine residue

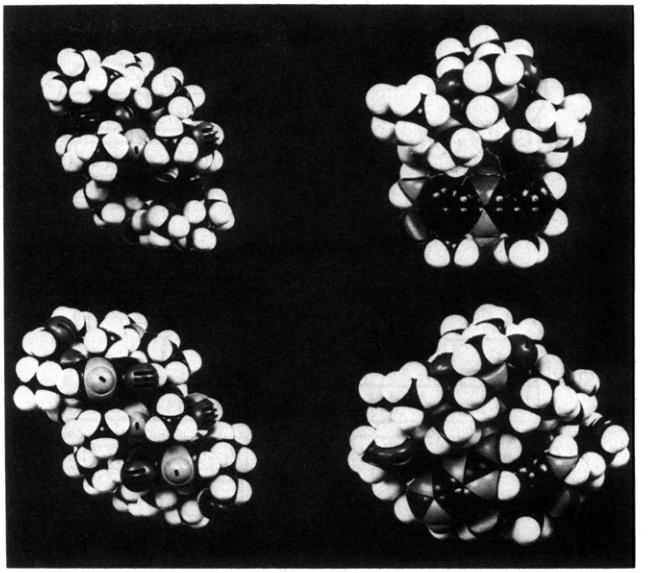

FIGURE 7: Corey–Pauling–Koltun space filling models of the actinomycin D–deoxyguanosine complex as observed in the solid state by Sobell *et al.* (1971). Top left: front view of actinomycin D. The 4- and 6-methyl groups project out of the plane toward the viewer. Top right: actinomycin D viewed from above the plane of the chromophore. Bottom left: front view of the actinomycin D–$(deoxyguanosine)_2$ complex showing the deoxyguanosine molecules stacked on each side of the phenoxazone ring. Bottom right: top view of the actinomycin D–$(deoxyguanosine)_2$ complex showing the molecule bound in the 6-binding site. This figure has been reproduced, with permission, from a review article by Sobell (1973).

located near the quinoid portion of the chromophore and one guanine residue on the benzenoid side of the ring. Actinomycin D exists predominantly in the dimer form for the range of concentrations used in the present experiments. In the dimer form two of the four possible binding sites for dGMP are on the inside of the stacked chromophores and are not available for binding nucleotides. Since the dimerization consists of stacking the chromophores with one chromophore inverted with respect to the other, one 4-binding site and one 6-binding site will be located between the stacked chromophores (Figure 8).

The data in Figure 4 for the titration with dGMP show that the 4-CH_3 resonance initially moves *upfield* and then moves downfield as the actinomycin D dimer is disrupted. The initial upfield shift of the 4-CH_3 can only be interpreted as association of some of the added dGMP with the dimerized form of actinomycin D. Such an interaction is not unreasonable since the two available binding sites in the dimer are relatively free from steric interference with the peptide chains of the second actinomycin D molecule in the dimer. In order to form the actinomycin D–$(dGMP)_2$ complex the nucleotide must effectively compete with the dimerization process. The binding sites for dGMP are located on opposite sides of the chromophore and thus the formation of the actinomycin D–$(dGMP)_2$ complex must effectively eliminate the possibility of any further interaction between actinocyl rings. The optical experiments indicate that the association constant of actinomycin D and dGMP is $\sim 9 \times 10^{-3}$ M^{-1} at 3° (Gellert *et al.*, 1965), while the dimerization constant is $\sim 3 \times 10^{3}$ M^{-1} at the same temperature. A consideration of these two equilibrium constants shows that the nucleotide complex formation is favored but that dimerization should be a factor until a significant excess of nucleotide is added. However, the observation that the 4-CH_3 chemical shift ceases to change when the ratio

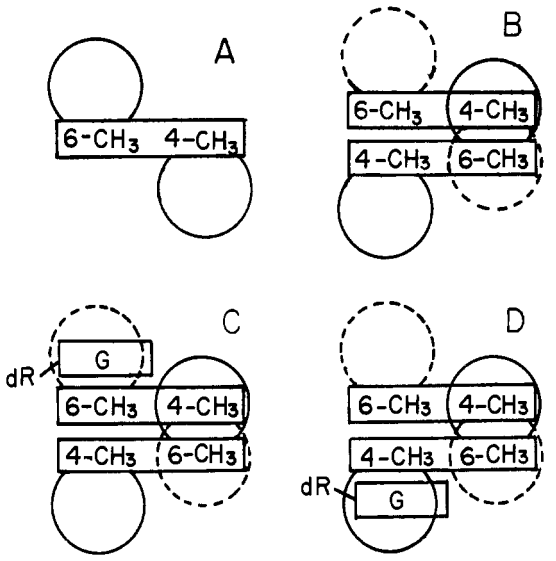

FIGURE 8: Schematic illustration of actinomycin D: (A) actinomycin D monomer; (B) actinomycin D in the inverted dimer form; the dashed circles represent the pentapeptide rings that are on the back side of the complex; (C) actinomycin D dimer with a deoxyguanosine bound in the 6-binding site; (D) actinomycin D dimer with a deoxyguanosine molecule bound in the 4-binding site.

of [dGMP]/[actinomycin D] = 2 indicates that the dimer is destabilized by the presence of the first dGMP and that the dimerization constant is reduced during the titration.

Interaction with GMP. The interaction of actinomycin D and GMP provides additional evidence that the geometry proposed above for dGMP is correct. In Figure 7 the 2′ proton of one of the dGMP molecules is located near the H-8 proton. Substitution of a hydroxy group at the 2′ position would be expected to influence the chemical shifts of the H-7 and H-8 protons. As shown in Figure 3, the initially equivalent H-7 and H-8 protons are split into a well-resolved AB pattern consistent with the placement of a deshielding OH group nearer the H-8 than the H-7 proton. We therefore assign the upfield doublet in the AB pattern to the H-7 proton and the downfield doublet to the H-8 proton. Presumably the steric interference with the 2′-hydroxy group slightly reduces the amount of overlap of the guanine ring with the chromophore of actinomycin D forcing the guanine ring slightly toward the H-7 and H-8 protons. This would increase the shielding of the H-7 and H-8 protons but the 2′-hydroxy group presumably shifts the H-8 proton back downfield resulting in a small net downfield shift relative to that for dGMP.

The presence of a 2′-hydroxy group on the nucleotide bound in the 4-binding site would result in a great deal of steric and electrostatic repulsion due to the presence of the 2-amino group on the chromophore (Figure 1). This repulsion can be reduced and additional stability gained by placing the GMP in a position that allows the formation of a hydrogen bond between the 2′-hydroxy group and the 3-carbonyl oxygen on the chromophore. The new orientation would place the guanosine base slightly closer to the 4-CH_3 group, consistent with the observed upfield shift of the 4-CH_3 resonance relative to that for dGMP.

A change in the relative orientation of the GMP bound in the 4- and 6-binding sites is confirmed by the observation that the two MeVal N–CH_3 and the two Thr CH_3 groups exhibit different chemical shifts upon addition of GMP. One of the MeVal N–CH_3 chemical shifts is very similar to that for dGMP. The orientation of the GMP in the 6-binding site has been presumed to change very little from that for dGMP. Therefore, the MeVal N–CH_3 curve that is similar to the curve observed in the dGMP titration is assigned to the MeVal N–CH_3 near the 6-binding site. The second MeVal N–CH_3 resonance changes very little upon addition of GMP. This is consistent with the new geometry proposed for the 4-binding site. The guanosine base is moved slightly toward the 4-CH_3 and away from the MeVal N–CH_3, resulting in a reduction of the induced chemical shift of this group.

The Thr CH_3 chemical shifts are consistent with this picture as well. One Thr CH_3 is shifted ~0.11 ppm farther downfield with GMP than dGMP because the 2′-hydroxy group in the 6-binding site is positioned near the Thr CH_3 group. The downfield resonance is therefore assigned to the Thr CH_3 group near the 6-binding site. The chemical shift of the Thr CH_3 near the 4-binding site is very similar to that measured for dGMP. This is expected if the 2′-hydroxy group in the 4-binding site is located in a position that allows hydrogen bond formation to the 3-carbonyl on the chromophore.

It very important to note that the shapes of the 6-CH_3 curves for both dGMP and GMP are essentially the same. This strongly indicates that the association constant for GMP binding to the 6-binding site is about equal to the dGMP binding constant. The present experiments are not particularly good for determining the values of the strong binding constants ($K \gtrsim 1 \times 10^3$) because the fairly concentrated actinomycin D solutions required for the experiments result in almost total binding of the nucleotide whenever the ratio of nucleotide to actinomycin is less than 2:1. The self-association of actinomycin D also complicates the analysis of the spectra in terms of nucleotide binding constants. We estimate that both binding constants [K_6(dGMP) and K_6(GMP)] are $> 3 \times 10^3$ M^{-1}.

The continuing change of the 4-CH_3 chemical shift when [GMP]/[actinomycin D] > 2 indicates that the association constant for GMP in the 4-binding site is reduced compared to dGMP. Optical experiments that monitor the change in the visible spectrum of actinomycin D upon addition of GMP (Behme and Cordes, 1965) indicate that the association constant for GMP is 0.24×10^3 M^{-1} compared to a dGMP binding constant of 1.8×10^3 M^{-1}. Using these values we calculate that a significant portion of the GMP will remain unbound when [GMP]/[actinomycin D] = 2. We observe this for GMP binding to the 4-binding site but not the 6-binding site. Thus the change in the visible spectrum of actinomycin D results primarily from nucleotides bound in the 4-binding site. The nmr results demonstrate a potential limitation of the optical experiments. As discussed previously (Krugh, 1972), the change in the optical spectra may result from either or both of the guanine nucleotides complexing with actinomycin D. The nmr experiments monitor binding of each of the GMP molecules, while the optical spectra primarily monitor the weak binding site for GMP (*i.e.*, the 4-binding site).

Interaction with dAMP. The interaction of actinomycin D and 5′-dAMP also provides some very important information (Figure 6). The most striking observation is that the shape of the 6-CH_3 curve is almost identical with the dGMP titration, indicating that the association constant for the binding of dAMP to the 6-binding site is $\gtrsim 3 \times 10^3$ M^{-1}. A comparison of the 4-CH_3 curve with the 6-CH_3 curve shows that K_4(dAMP) is much less than K_6(dAMP). The results from previous optical studies led several workers to conclude that the binding constant for dGMP is much larger than the binding constant for dAMP. The present results indicate that this statement is only half true. The values of the binding constants must be deter-

mined from careful spectral measurements at lower actinomycin D concentrations.

The induced upfield shift for the 6-CH_3 group in the actinomycin D–$(dAMP)_2$ complex is 34 Hz, identical with the value for the dGMP complex. The H-7 and H-8 protons are shifted upfield somewhat less in the dAMP complex as compared to the dGMP complex, while the MeVal N–CH_3 groups are shifted upfield an additional 10 Hz in the dAMP complex. The induced chemical shifts for the protons associated with 6-binding site are consistent with a molecular geometry very similar to that presented for dGMP bound in the 6-binding site of actinomycin D. In this configuration only one hydrogen bond may be formed between the pentapeptide ring and the nucleotide (*i.e.*, N-3 of adenine to the N–H of the L-threonine since dAMP does not have a 2-amino group. Two hydrogen bonds could be formed if the orientation of the dAMP molecule is inverted, but the resultant geometries do not appear to be consistent with the observed trends in the chemical shifts. We feel that the observed chemical shifts are best interpreted in terms of a geometry similar to that observed for the dGMP complex, except that the adenine base is rotated with respect to the phenoxazone ring to form a strong hydrogen bond between N-3 and the L-threonine N–H group. This places the 2′ proton between the H-7 and H-8 protons if the dAMP is an anti conformation. The MeVal N–CH_3 chemical shifts also clearly illustrate that the two dAMP binding sites have very different binding constants. One curve monitors the strong binding site and the second MeVal N–CH_3 curve monitors the weak binding site. The lack of a measurable chemical-shift difference for the two Thr CH_3 resonances is probably due to the relatively small chemical-shift changes involved. The doublet character of the resonances requires chemical-shift differences of the order of the coupling constant (~5 Hz) before two separate resonances could be resolved.

Interaction with AMP. In the AMP titration the chemical shift of the 6-CH_3 group continued to change until the ratio of AMP to actinomycin D was $\cong 5$, whereas this shift was invariant at nucleotide/drug ratios $\gtrsim 2$ in the dAMP titration. This indicates that either K_6(AMP) is much less than K_6(dAMP) or that the AMP bound in the 4-binding site influences the chemical shift of the 6-CH_3 group. The two binding constants are probably not equal but we feel that the slow change in the chemical shift of the 6-CH_3 group (Figure 7) for nucleotide/drug ratios ≥ 2.5 results from the cross shielding of the 6-CH_3 by an AMP bound in the 4-binding site. This is supported by the observation that the chemical shifts of both the 4-CH_3 and the 6-CH_3 groups reach their final value when [AMP]/[actinomycin D] $\cong$ 5. The chemical shifts of one of the MeVal N–CH_3 groups and the H-7 and H-8 protons do not change for nucleotide/drug ratios $\gtrsim 2$, which provides additional evidence for the cross shielding of the 6-CH_3 group by an AMP bound in the 4-binding site.

Cross shielding of the 6-CH_3 group by a nucleotide bound in the 4-binding site appears to be negligible in both the dGMP and GMP titrations, which places restrictions on the orientation of the guanine ring with respect to the chromophore. The present data do not allow us to directly determine if a nucleotide bound in the 6-binding site affects the shift of the 4-CH_3 group. The observation of cross shielding is not surprising and we discuss it primarily to emphasize that an initial analysis of the shape of the 6-CH_3 curves in Figure 6 would indicate that K_6(AMP) $\ll$ K_6(dAMP). When the cross shielding is considered and the MeVal N-CH_3 and the H-7 and H-8 curves are compared, the two binding constants appear to be approximately equal.

It is interesting that the H-7 and H-8 protons remain equivalent in the AMP titration. The Thr CH_3 groups also remain equivalent and therefore the 2′-hydroxy group on the AMP molecule bound in the 6-binding site is not located in the area between the Thr CH_3 and the H-8 proton as proposed for the GMP complex. Both the H-7 and H-8 protons are shifted upfield less in the AMP complex compared to the dAMP complex, which suggests that the 2′-hydroxy group is located close to both the H-7 and H-8 protons and deshields them equally.

Summary

The present experiments have provided several new and important observations relevant to a description of the actinomycin D–DNA interaction. We have confirmed that actinomycin D forms a complex with *two* 5′-dGMP nucleotides as suggested by previous studies (Arison and Hoogsteen, 1970; Krugh, 1972) and as observed in the solid state by Sobell and coworkers (1971). The present data also conclusively show that the association of dGMP and actinomycin D results from a "stacking" of the guanine bases with the phenoxazone ring of the actinomycin D which agrees with previous nmr results (Danyluk and Victor, 1970; Arison and Hoogsteen, 1970). We have determined the orientation of the guanine and adenine ring with respect to the phenoxazone ring and we have found that for dGMP the geometry observed in the solid state complex with deoxyguanosine (Sobell *et al.*, 1971) is consistent with our results. Additional experiments are required to determine the stereochemistry of the pentapeptide rings but the chemical shifts changes of the Thr CH_3, the MeVal N–CH_3, and the sarcosine N–CH_3 groups in forming the actinomycin D–$(dGMP)_2$ complex are consistent with the peptapeptide groups having a conformation in solution similar to that observed in the solid state complex. All the purine nucleotides studied will complex with actinomycin D, in agreement with several previous optical studies. However, the previous optical studies were only half correct in stating that the GMP binding constant is much smaller than the dGMP binding constant. The present data clearly indicate that both GMP and dGMP will bind to the 6-binding site with a binding constant $\geq 3 \times 10^3$ M^{-1}, while the GMP binding constant for the 4-binding site is much less than the dGMP binding constant. This observation not only illustrates the specificity of the 4-binding site but it also shows that the changes in the optical spectra observed upon addition of nucleotides are primarily a result of the perturbations of the electronic structure of the quinoid portion of the chromophore. As a result, the visible spectral titrations primarily monitor the 4-binding site. A similar situation was found with the adenine nucleotides where the dAMP binding to the 6-binding site was much stronger than the binding to the 4-binding site. The optical spectra had indicated only the presence of the weak binding site. A subsequent paper will discuss the interactions of actinomycin D and deoxydinucleotides (Krugh and Neely, 1973) in which we have used nmr to confirm the model proposed for complex formation (Krugh, 1972). We shall defer the discussion of the obvious biological significance of the present results until the dinucleotide results are presented since the dinucleotides have proven to be an excellent model system in the investigation of the actinomycin D–DNA interaction (Krugh, 1972).

Added in Proof

Schara and Müller (1972) have recently investigated the

complexes of actinomycin C_3 with mononucleotides, deoxydinucleotides, and an oligonucleotide using optical spectral technique. These authors found that AMP formed a 2:1 complex with actinomycin C_3, consistent with the present results for actinomycin D. However, they report finding a 1:1 stoichiometry for the actinomycin C_3–dAMP complex. The present results indicate that both dAMP and AMP form 2:1 complexes with actinomycin D.

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Ultraviolet Irradiation of the Components of the Wheat Embryo *in Vitro* Protein Synthesizing System†

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ABSTRACT: In order to evaluate the sensitivities of the biological activities of plant RNA molecules to ultraviolet (uv) light, various components of protein synthesis were isolated from wheat embryos, irradiated, and then tested for their ability to support amino acid incorporation in an *in vitro* system. Messenger RNA was the component most sensitive to uv irradiation. The inactivation cross sections for two messengers, tobacco mosaic virus (TMV)-RNA and polyuridylic acid, were 50×10^{-5} and 68×10^{-5} mm²/erg, respectively, compared to cross sections for wheat ribosomes, transfer RNA, and S100-DEAE (mixed enzymes without transfer RNA activity) of 7.0×10^{-5}, 1.0×10^{-5}, and 2.2×10^{-5} mm²/erg, respectively. Wheat ribosomes were significantly more uv sensitive than *Escherichia coli* ribosomes. The ability of TMV-RNA to cause infection in tobacco was eight times more uv sensitive than was its messenger activity.

Short-wave ultraviolet radiation (uv[1]) strongly interacts with RNA and chemically alters its constituent pyrimidine nucleotides, forming pyrimidine hydrates, cyclobutane-type pyrimidine dimers, and other, uncharacterized photoproducts. Uv also disrupts the biological activities of RNA molecules. Previous studies of tRNA in amino acid activating assays and of tRNA, mRNA, and ribosomes in amino acid incorporating assays have indicated the sensitivity of these RNA species to uv-induced changes in their structures (Grossman, 1962; Wacker *et al.*, 1962; Swenson and Nishimura, 1964; Harriman and Zachau, 1966; Kagawa *et al.*, 1967; Ottensmeyer and Whitmore, 1968; Tokimatsu *et al.*, 1968; Wada *et al.*, 1968; Aoki *et al.*, 1969; Eker and Berends, 1970; Remsen and Cerutti, 1972). The studies have shown that uv has both inactivating and, in some cases, mutagenic effects on RNA.

Uv radiation may be a particularly significant stress to the protein synthetic system in higher plants, since plants, as obligatory photosynthetic organisms, are often exposed to relatively high doses of uv light from the sun. The idea that plant RNA-containing components may suffer from uv-induced inactivation is supported by the fact that certain plants possess mechanisms for photoreactivating uv-inactivated RNA viruses and RNA-viral genomes (Kleczkowski, 1971; Murphy and Gordon, 1971). However, this idea has not been firmly established. For one thing, there are no reports of photoreactivation of uv-damaged cellular (nonviral) RNA. For another, there is little information available about the uv sensitivity of plant RNA species. The uv-irradiation studies mentioned above have emphasized procaryotic systems. The effects of uv on plant ribosomes or on mes-

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[1] Abbreviations used are: uv, short-wave ultraviolet radiation (190–300 nm); TMV, tobacco mosaic virus.