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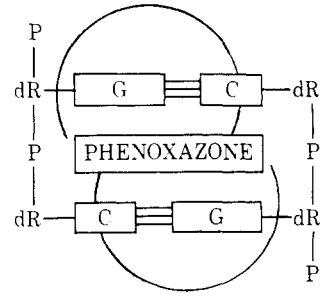
Peptide Antibiotic–Dinucleotide Interactions. Nuclear Magnetic Resonance Investigations of Complex Formation between Actinomycin D and d-pGpC in Aqueous Solution†

Dinshaw J. Patel

ABSTRACT: This study reports on the high resolution ^{1}H and ^{31}P nuclear magnetic resonance (nmr) spectra of 1:2 actinomycin D (Act-D)–d-pGpC in aqueous solution. A 1.6 ppm downfield ^{31}P shift of the internucleotide phosphate of d-pGpC on binding to Act-D in aqueous solution supports the Sobell–Jain proposal (H. M. Sobell, and S. C. Jain (1972), *J. Mol. Biol. 68*, 21) that the antibiotic intercalates between GC and CG Watson–Crick base pairs. The proton chemical shift changes for the Act-D and d-pGpC resonances on complex formation compare favorably with those predicted to arise from the ring currents and magnetically anisotropic groups of the stacked residues in the Sobell–Jain model of the complex. The guanine 2-amino exchangeable proton(s) shift downfield on complex formation consistent with their participation in hydrogen bond formation in aqueous solution.

Ribonucleic acid synthesis is inhibited when the peptide antibiotic actinomycin D (Act-D),[1] an antitumor agent, binds to double helical DNA (for a review, see Reich and Goldberg, 1964). The structure of the Act-D–DNA complex has been suggested to involve a hydrogen-bonding recognition between the guanine 2-amino proton (G–NH_2) and an acceptor group on Act-D on the basis of chemical studies of deoxy polynucleotides possessing or lacking the G–NH_2 group (Reich *et al.*, 1962). On the other hand, an intercalation model has been proposed based on spectroscopic, hydrodynamic, and kinetic studies (Müller and Crothers, 1968) and on the sedimentation behavior of supercoiled closed circular DNA in the presence of Act-D (Waring, 1970).

A detailed stereochemical model for the binding of Act-D to DNA has been proposed (Sobell and Jain, 1972; Sobell, 1973) on the basis of an X-ray study of the 1:2 Act-D–d-G complex in the crystal (Sobell *et al.*, 1971; Jain and Sobell, 1972). Act-D is suggested to intercalate between base-paired d-pGpC sequences such that the phenoxazone ring is stacked between GC and CG Watson–Crick base pairs. The complex is further stabilized by strong intermolecular hydrogen bonds involving the guanine 2-amino group and hydrophobic interactions between groups on the sugar and peptide rings. The pseudotwofold symmetry of the 1:2 Act-D–d-pGpC results from the twofold symmetry of the dinucleotide duplex coinciding with the pseudotwofold symmetry of the Act-D molecule.



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[1] Abbreviation used is: Act-D, actinomycin D.

TABLE I: ^{31}P Chemical Shifts of the Internucleotide and Terminal Phosphorus Atoms of 0.05 M d-pGpC in D_2O Solution at 8° as a Function of pH.

	Chemical Shifts (ppm)[a] pH 5.55	pH 7.88	Ionization Shifts (ppm)
Internucleotide	−0.54	−0.48	0.06 upfield
Terminal	+0.90	+3.98	3.08 upfield

[a] Referenced relative to 16% phosphorus acid in D_2O.

The binding of dinucleotides to actinomycin D has been investigated from optical spectra titration studies (Krugh, 1972; Schara and Müller, 1972). Dinucleotides of the sequence d-pCpG (where X is any of the four nucleotide bases) bind Act-D with the same efficiency as d-pG while the sequences d-pGpC exhibits the strongest binding constants to the antibiotic. Krugh and Neely (1973) have extended their optical results to proton nmr studies in D_2O. Dinucleotides of the sequence d-pXpG and d-pGpX were added to actinomycin D in aqueous solution and the complexation shifts were analyzed in terms of the Sobell–Jain model.

Experimental Section

d-pGpC was purchased from Collaborative Research and was passed through a Chelex column prior to use. Actinomycin D was purchased from Merck. ^{13}C and ^{31}P nuclear magnetic resonance (nmr) spectra were run on a Varian XL-100 spectrometer interfaced to a Nicolet 1080 computer in the Fourier transform mode. The spectrometer was locked to the deuterium resonance of solvent D_2O. ^{31}P chemical shifts at 40.51 MHz were referenced relative to external 16% phosphoric acid in D_2O. ^1H nmr spectra were run on a Varian 300-MHz superconducting spectrometer operating in either the field or frequency mode. ^1H chemical shifts were referenced to either internal 2,2-dimethyl-2-silapentane-5-sulfonic acid in D_2O or to the HOH signal in H_2O. The chemical shift of the HOH resonance relative to 2,2-dimethyl-2-silapentane-5-sulfonic acid as a function of temperature was previously calibrated. The spectra were time averaged where necessary on a Nicolet 1074 computer containing 4K channels. Spin decoupling was undertaken in the frequency sweep mode utilizing a Varian decoupler with its own frequency synthesizer. CD spectra were run on a Cary 61 spectrometer.

For calculating line shapes of exchanging species, use was made of a computer program devised by M. Cocivera which stimulates chemical exchange processes for a maximum of six sites. The input parameters are the chemical shifts, population of exchanging sites, and the line widths in the absence of exchange.

Results

A. Intercalation Site. The dinucleotide d-pGpC contains a terminal phosphorus atom at the 5′ end and an internucleotide phosphorus atom. Figure 1-I presents the ^{31}P nmr spectra of d-pGpC in D_2O, 8° at two pH values with the chemical shifts referenced relative to external 16% phosphoric acid in D_2O. Two phosphorus atoms are observed at both pH values, one of which shifts upfield by 3 ppm on going from pH 7.88 to pH 5.55, while the other phosphorus chemical shift remains unchanged over this pH range (Table I). The terminal phosphate group of the dinucleotide titrates over this pH range, permitting assignment of the −0.48- and +3.98-ppm resonances to the terminal and internucleotide phosphorus atoms of d-pGpC in D_2O, pH 7.88, respectively.

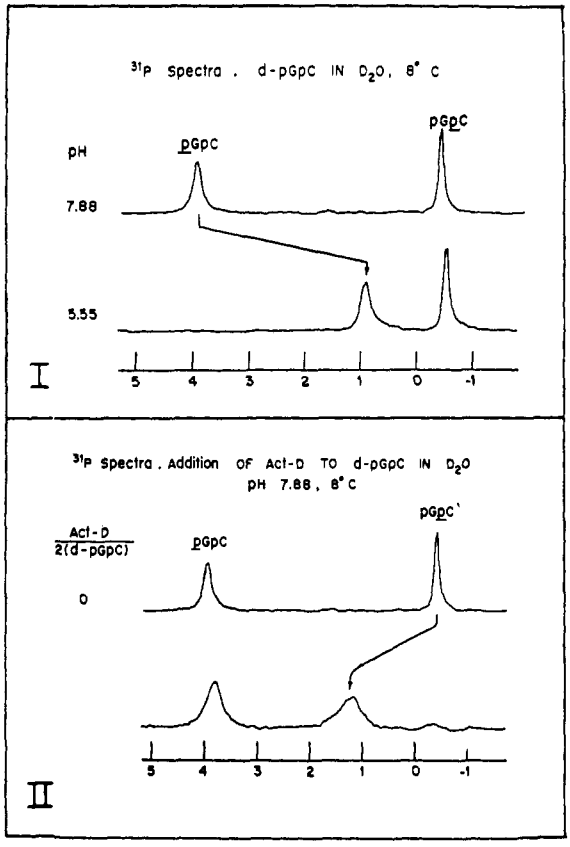


FIGURE 1: The 40-MHz ^{31}P nmr spectra referenced relative to 16% phosphoric acid: (I) 0.05 M d-pGpC in D_2O, 8° at pH 7.88 and 5.55; (II) 0.05 M d-pGpC in D_2O, pH 7.88, 8° and 0.025 M 1:2 Act-D–d-pGpC in D_2O, pH 7.88, 8°.

The ^{31}P spectra of d-pGpC in the absence and presence of Act-D at pH 7.88, 8° in D_2O, are presented in Figure 1-II. The terminal phosphorus atom of d-pGpC moves upfield by 0.1 ppm on complex formation with Act-D, while the internucleotide phosphorus atom of d-pGpC shifts downfield by 1.7 ppm in the 1:2 Act-D–d-pGpC complex (Table II). This large downfield phosphorus chemical shift on complex formation was determined to be pH independent (pH 6.3–8.7) at 30° (Table III).

B. Complex Formation. The ^{31}P spectrum of a mixture of 1 equiv of Act-D and 4 equiv of d-pGpC in H_2O, pH 8 and 9°, is shown in Figure 2. While the terminal phosphorus atom

TABLE II: ^{31}P Chemical Shifts of the Internucleotide and Terminal Phosphorus Atoms of d-pGpC and 1:2 Act-D–d-pGpC in D_2O Solution, pH 7.88, 8°.

	d-pGpC[b] (ppm)[a]	1:2 Act-D–d-pGpC[c] (ppm)[a]	Complexation Shifts (ppm)
Internucleotide	−0.48	+1.20	1.68 downfield
Terminal	+3.98	+3.86	0.12 upfield

[a] Referenced relative to 16% phosphorus acid in D_2O. [b] 0.05 M. [c] 0.025 M.

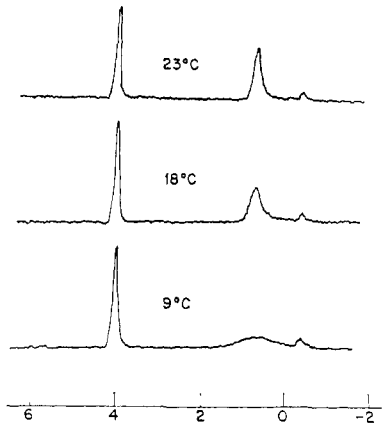


FIGURE 2: The 40-MHz ^{31}P nmr spectra of 1:4 Act-D–d-pGpC in 0.1 M NaCl–0.01 M EDTA–H_2O (pH 8) as a function of temperature.

exhibits a narrow resonance, the internucleotide phosphorus atom is considerably broadened. On raising the temperature, this resonance undergoes exchange narrowing. The data suggest that the exchange process

$$\begin{aligned} &2d\text{-}pGpC + 1:2\ \text{Act-D–d-pGpC} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \\ &2\text{d-pGpC} + 1:2\ \text{Act-D–}d\text{-}pGpC \end{aligned} \quad (1)$$

exhibits intermediate rates on the nmr time scale at 9°. The exchange process was simulated with the Cocivera computer program for two sites of equal population separated by 63 Hz (*i.e.*, 1.6 ppm at 40 MHz) undergoing rapid exchange with lifetime broadening. The line width in the absence of exchange was set to the value observed for the terminal phosphorus, *i.e.*, 4 Hz. The average lifetimes, $\tau = \tau_1\tau_2/\tau_1 + \tau_2$ determined by computer simulation are presented in Table IV. The exchange process exhibits average lifetimes of 2.6 and 0.23 msec at 9 and 27°, respectively (Table IV).

The rate constants, k_1 and k_{-1}, for process 1 are equal to $^1/_2\tau$, permitting an estimation of the free energy of activation from the relationship

$$\Delta F^{\pm} = 2.303RT(10.319 + \log T - \log k_1)$$

The temperature dependent free energies of activation $\Delta F^{\pm}$ are summarized in Table IV. The value at 9° is $\Delta F^{\pm} = 13.60$ kcal/mol.

C. Stacking Orientations in the Complex. The proton nmr spectrum between 0 and 8 ppm relative to standard 2,2-dimethyl-2-silapentane-5-sulfonic and of the 1:2 complex of Act-D with d-pGpC in D_2O has been recorded at pH 5.8 at 26°. The chemical shifts are summarized in Table V, which includes selective chemical shifts for nonaggregated Act-D in aqueous solution (Angerman *et al.*, 1972) and the chemical shifts of the base and sugar carbons of d-pGpC in aqueous solution. The phenoxazone CH_3 proteons at 2.61 and 2.23 ppm are upfield shifted by 0.46 and 0.39 ppm, respectively, on complexation. The phenoxazone benzenoid ring protons (positions 7 and 8) at 7.56 ppm are upfield shifted by 0.23 and 0.68 ppm. The CH_3 protons of the side chain of L-Thr at 1.38 ppm in Act-D undergo an 0.15-ppm downfield shift. While the cytosine H-6 proton is unaffected, the guanine H-8 and cytosine H-5 protons of d-pGpC are shifted 0.2 ppm upfield and downfield, respectively, on complex formation. The two H-1′ protons of d-pGpC undergo 0.25-ppm shifts in opposite directions on addition of Act-D.

Comparison of the aromatic regions of the 1:2 Act-D–d-pG and 1:2 Act-D–d-pGpC complexes indicates that in the former the phenoxazone H-6,7 protons are a barely resolved AB

TABLE IV: Kinetic Parameters for the Exchange Process (eq 1) as Followed by ^{31}P Nmr of the Internucleotide Phosphorus Atom of d-pGpC.[a]

Temp (°C)	Obsd Width (Hz)	τ (msec)	$\Delta F^{\pm}$ (kcal)
9	46	2.6	13.60
18	13.1	0.72	13.30
23	9.4	0.43	13.25
27	6.9	0.23	13.05

[a] $\Delta S^{\pm} = 29.2$ eu; $\Delta H^{\pm} = 21.8$ kcal.

TABLE III: ^{31}P Chemical Shifts of the Internucleotide and Terminal Phosphorus Atoms of d-pGpC and 1:2 Act-D–d-pGpC in D_2O Solution at 30° as a Function of pH.

pH	d-pG*p*C[b] (ppm)[a]	1:2 Act-D–d-pG*p*C[c] (ppm)	Downfield Shifts on Complexation
6.32	−0.27	+1.24	1.51
7.08	−0.30	+1.30	1.60
7.88	−0.27	+1.27	1.54
8.70	−0.27	+1.30	1.57

[a] Referenced relative to 16% phosphoric acid in D_2O. [b] 0.05 M. [c] 0.025 M.

TABLE V: Proton Chemical Shifts in Ppm Relative to Standard 2,2-Dimethyl-2-silapentane-5-sulfonic Acid for the Resonances of Act-D, d-pGpC, and 1:2 Act-D–d-pGpC Complex in Aqueous Solution.

Residue		Act-D[a] (4°)	d-pGpC[b] (pH 5.8, 26°)	1:2 Act-D–d-pGpC[c] (pH 5.8, 26°)
L-*N*-MeVal	CH_3	0.82, 0.99		0.79, 0.96
	N-CH_3	3.01, 3.05		2.97
	H^{β}	2.55		
	H^{α}	3.40		
Sar	*N*-CH_3	2.90		2.89
	H^{α}	3.70, 3.77		3.63, 3.67
D-Val	CH_3	0.85, 1.10		0.89, 1.12
	H^{β}	2.15		
L-Thr	CH_3	1.38		1.53
	H^{β}	5.30[d]		5.29
Phenoxazone	H-7,8	7.56[e]		7.33, 6.88
	CH_3-4,6	2.61,[e] 2.23[e]		2.15, 1.84
Purine	H-8		8.09	7.86
Pyrimidine	H-5		5.82	6.07
	H-6		7.77	7.78
Sugar	H-1′		6.28, 6.21	6.50, 5.95

[a] The concentration and pH of this solution was not reported (Angerman *et al.*, 1972). [b] 0.04 M. [c] 0.02 M. [d] This investigation. [e] Chemical shifts reported for the Act-D monomer at pH 7.23, 19° (Angerman *et al.*, 1972).

quartet at 7.3 ppm while in the latter these protons exhibit an AX pattern separated by 0.45 ppm (Figure 3). This result has been independently observed by Krugh and Neely (1973).

D. Hydrogen Bonding. From the mononucleotide data of Raszka and Kaplan (1972), the G–NH_2 and C–NH_2 protons should exhibit narrow and very broad spectra, respectively, in the spectrum of d-pGpC in H_2O at 30°. On lowering the temperature toward 0°, the two C–NH_2 protons should be observable as separate narrow resonances.

(i) Guanine 2-Amino Protons (G-NH_2). The spectra of d-pGpC in H_2O (pH 5.8) have been recorded as a function of temperature. From an investigation of the spectrum of d-pGpC in H_2O and D_2O, the one proton triplets at 6.1–6.3 ppm are assigned to the nonexchangeable H-1′ protons of the sugar rings, the one proton doublet at ~5.8 ppm to the nonexchangeable H-5 proton to cytosine, and the two proton exchangeable resonances between 6.3 and 6.5 ppm are assigned to the G–NH_2 protons.

The proton nmr spectrum between 5.5 and 7.0 ppm of d-pGpC on gradual addition of Act-D in H_2O (pH 5.8) at 28 and 58° is shown in Figure 4. At 28°, proton nmr spectra are presented at 0, 0.1, and 0.3 equiv of Act-D per 2 equiv of d-pGpc (Figure 4). The spectra broaden significantly with increasing Act-D concentration and the chemical shifts cannot be readily followed. At 58°, proton nmr spectra are presented for 0, 0.4, and 0.65 equiv of Act-D per 2 equiv of d-pGpC (Figure 4). The G–NH_2 exchangeable protons are observed to shift downfield as average resonances with increasing Act-D concentration at this temperature. The H-1′ sugar protons and cytosine H-5 proton of d-pGpC also shift as average resonances with increasing Act-D concentration.

(ii) Difference Spectra (H_2O vs. D_2O) for 1:2 Act-D–d-pGpC Complex. The types and numbers of exchangeable protons in the 1:2 Act-D–d-pGpC complex are G–NH_2(4), C–NH_2(4), G–NH(2), A–NH_2(2), D-Val H^N(2), and L-Thr H^N(2), where H^N signifies the peptide protons of D-Val and L-Thr.

Difference spectra (H_2O *vs.* D_2O) for the 1:2 complex in aqueous solution at pH 6 are presented in Figure 5 and for spectra I and II recorded at 26 and 5°, respectively. The guanine H-8 and cytosine H-6 nonexchangeable protons (area 4 protons) between 7.8 and 7.9 ppm and the phenoxazone H-6 and H-7 nonexchangeable protons (area 1 proton each) at 7.3 and 6.9 ppm in the proton nmr spectrum of the 1:2 complex in D_2O, pH 6 at 26° (Figure 5, spectrum I) and 5° (Figure 5, spectrum II) are used for area calibration.

The exchangeable resonances between 8 and 8.3 ppm exhibit an area of ~5 protons at 26° (Figure 5, spectrum I) and ~4 protons at 5° (Figure 5, spectrum II) in the difference spectrum of the 1:2 Act-D–d-pGpC complex. The 4 peptide (H^N) protons of D-Val and L-Thr account for the observable areas at both temperatures. In the difference spectrum of the 1:2 Act-D–d-pGpC complex at 26°, there is an exchangeable resonance at 7.1 ppm with area ~3.5 protons (Figure 5, spectrum I). This resonance was assigned to the G–NH_2 protons from the investigation of the gradual addition of Act-D to d-pGpC in H_2O solution (Figure 4). On lowering the temperature to 5°, ~11 exchangeable protons are observed between 7.2 ± 0.5 ppm in the difference spectrum (H_2O/D_2O) of 1:2 Act-D–d-pGpC in aqueous solution (Figure 5, spectrum II). The exchangeable resonances of G–NH_2 (four protons), C–NH_2 (four protons), and A–NH_2 (two protons) in the complex account for the observable areas in this region.

(iii) Chemical Shifts. The proton chemical shifts of the exchangeable 2-amino guanine protons (G–NH_2) in d-pGpC and 1:2 Act-D–d-pGpC in H_2O (pH 5.8) as a function of temperature are shown in Figure 6. An 0.7-ppm downfield shift is observed for the G–NH_2 protons of d-pGpC on complex formation with Act-D at 30°.

TABLE VI: ^1H Chemical Shifts and Temperature Coefficients of Exchangeable Resonances of d-pGpC and 1:2 Act-D–d-pGpC in H_2O, pH 5.8.

	Chemical Shifts at 26°	Temp Coefficients (ppm/deg)
d-pGpC[a]		
G-NH_2	6.40	6.8×10^{-3}
Complex[b]		
G-NH_2	7.08	18.7×10^{-3}
Thr-NH	8.23	3.2×10^{-3}
Val-NH	8.32	3.3×10^{-3}
	8.37	3.7×10^{-3}

[a] 0.05 M d-pGpC in H_2O, pH 5.8. [b] 0.012 M 1:2 Act-D–d-pGpC in H_2O, pH 5.8.

A temperature coefficient of 6.7×10^{-3} ppm/deg for the G–NH_2 protons d-pGpC in H_2O (pH 5.8) increases to 18.7×10^{-3} ppm/deg for these same exchangeable protons in the 1:2 Act-D–d-pGpC in H_2O (pH 5.8) (Figure 6).

A temperature dependence of the chemical shifts of the peptide protons in the 1:2 Act-D–d-pGpC complex in H_2O (pH 5.8) has been measured and is presented in Table VI. The D-Val and L-Thr peptide protons exhibit temperature coefficients of 3.5×10^{-3} and 3.2×10^{-3} ppm/deg, respectively.

Discussion

A. Conformation of Dinucleotides in Solution. Investigations of ApA, CpA, CpG, ApC, and GpC suggested that these ribodinucleoside phosphates occur in the extended conformation with the glycosidic bonds being anti for both residues (Barry *et al.*, 1972). By contrast, nmr and ORD experiments support a model of right-handed anti–anti partially stacked conformations of the same dinucleoside phosphates in solution (Ts'o *et al.*, 1969; Smith *et al.*, 1973; Warshaw and Cantor, 1970; Warshaw and Tinoco, 1965). The dinucleotide d-pGpC is assumed to exist in an equilibrium between stacked and open forms in solution.

B. Complex Formation. The proton and phosphorus nmr studies were undertaken by adding Act-D gradually to d-pGpC in aqueous solution. Beyond a 1:2 ratio of Act-D–

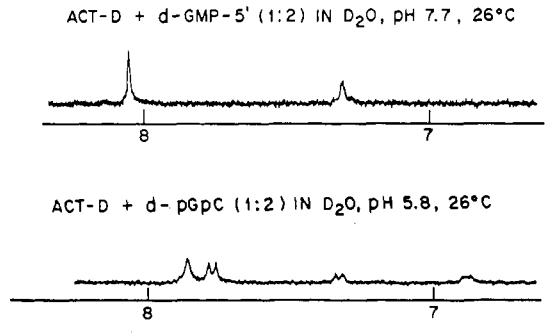


FIGURE 3: A comparison of the proton nmr spectra between 6.5 and 8.5 ppm for the complexes 1:2 Act-D–d-pG (top) and 1:2 Act-D–d-pGpC (bottom). The phenoxazone H-7,8 protons at 7.30 ppm in the mononucleotide complex are at 6.88 and 7.33 ppm in the dinucleotide complex.

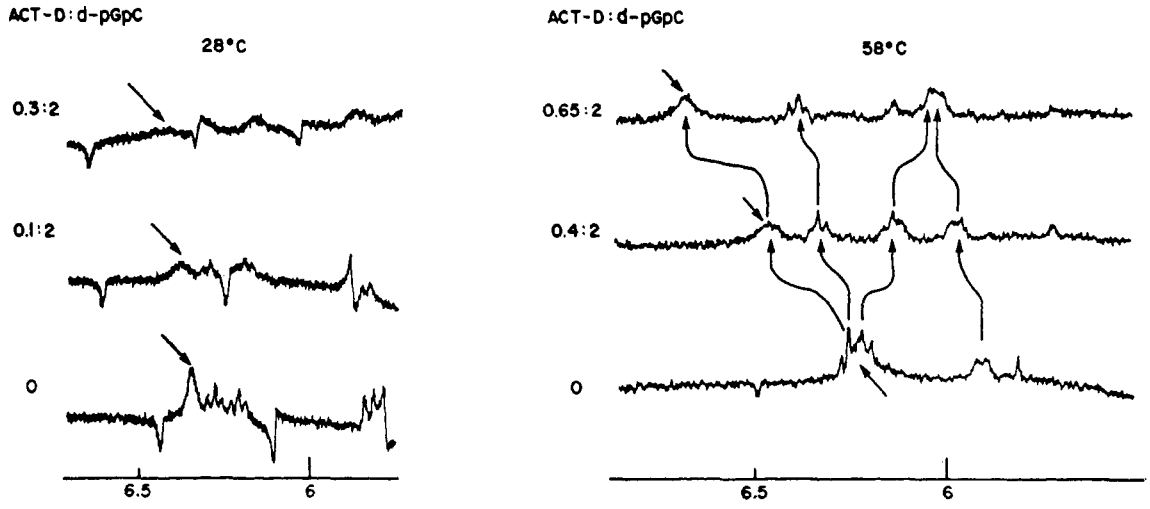


FIGURE 4: The 300-MHz ^1H nmr spectra of the gradual addition of Act-D to 0.025 M d-pGpC at 28 and 58°. The straight arrows designate the chemical shifts of the G–NH_2 protons.

d-pGpC, the antibiotic precipitated. Since d-pGpC is water soluble and the antibiotic sparingly so, the data suggest that the stoichiometry of the water-soluble complex is 1 equiv of antibiotic to 2 equiv of dinucleotide.

(*i*) *Intercalation Site.* For 1:2 Act-D–d-pGpC, the terminal and internucleotide phosphorus atoms would be distant from and in the plane of the phenoxazone ring, respectively (Sobell and Jain, 1972). On complex formation, the terminal phosphorus of d-pGpC undergoes an 0.1-ppm upfield shift while the internucleotide phosphorus atom undergoes a 1.7-ppm downfield shift (Figure 1-II, Table II). The 0.1-ppm upfield shift for the terminal phosphorus atom of d-pGpC on complex formation is of similar magnitude to that observed for the single phosphorus atom of d-pG on formation of the 1:2 Act-D–d-pG complex in aqueous solution (Patel, 1974).

The experimental ^{31}P downfield shift of 1.7 ppm for the internucleotide phosphate group cannot be accounted for by the contributions (<0.1 ppm) arising from the ring currents and magnetic anisotropic groups on the phenoxazone ring (Johnson and Bovey, 1958; Jackman and Sternhell, 1969). At this time no quantitative interpretation of this downfield shift can be presented. Since the phosphate group is charged medium effects on its chemical shift may be large. The internucleotide phosphate group is exposed to solvent water in the dinucleotide, while one side of it faces a hydrophobic surface of the phenoxazone residues in the complex. Alternately, a

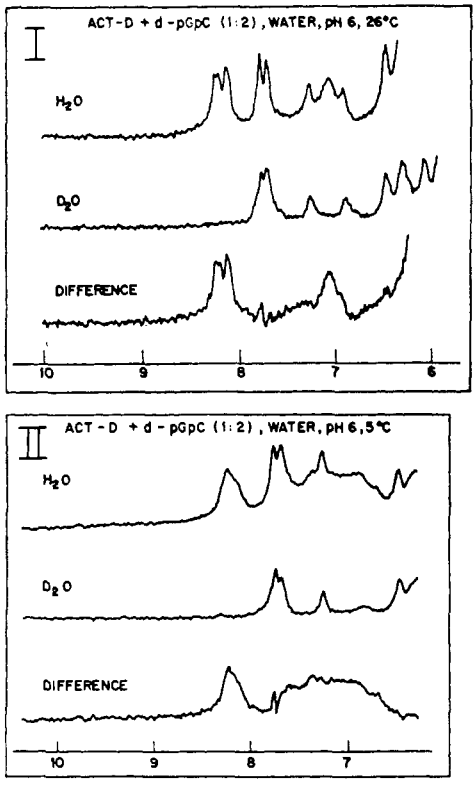


FIGURE 5: The 300-MHz (nonspinning) ^1H nmr difference spectra (H_2O *vs.* D_2O) of 0.025 M 1:2 Act-D–d-pGpC complex at pH 6. Spectra I are at 26° and spectra II at 5°.

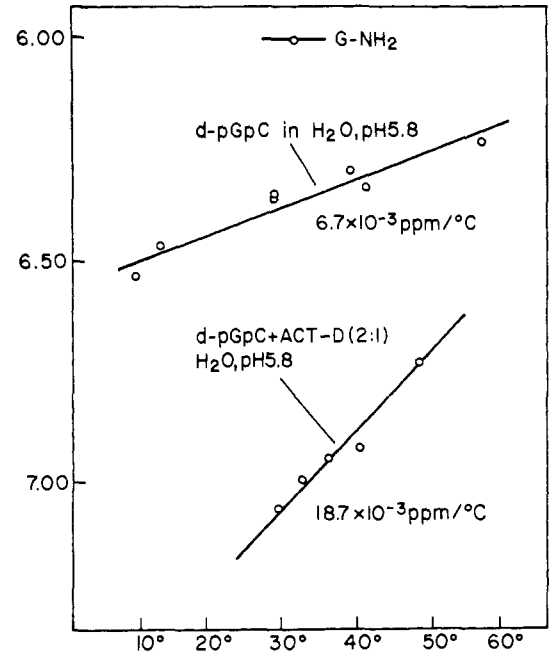
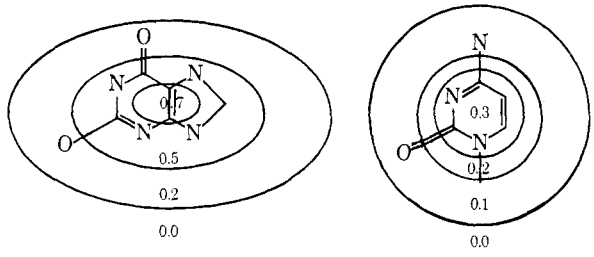


FIGURE 6: Temperature coefficients in ppm/deg of the G–NH_2 protons in the 300-MHz ^1H nmr spectra of 0.05 M d-pGpC in H_2O (pH 5.8) and 0.012 M 1:2 Act-D–d-pGpC in H_2O (pH 5.8).

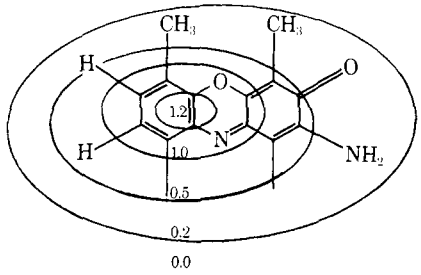
change in the P–O dihedral angles for the internucleotide phosphate group on intercalation could result in a chemical shift change on complex formation.

(*ii*) *Exchange Rates.* The proton nmr resonances of the dinucleotide can be followed on the gradual addition of Act-D to d-pGpC in aqueous solution (Figure 4). The observation of line broadening at 28° but not at 58° at pH 5.8 suggests intermediate and fast exchange at the two temperatures, respectively. The exchange process is associated with an activation free energy $\Delta F^{\ddagger} \sim 13$ kcal consistent with the observation of average resonances for the free and complexed forms.

C. Stacking Geometry in Solution. (*i*) *Ring Current Models.* Giessner-Prettre and Pullman (1970) have evaluated the ring current shifts in a plane parallel and at a distance of 3.4 Å from the guanosine purine ring. The upfield ring current contours in ppm are presented below.



Giessner-Prettre and Pullman (1970) have estimated the ring currents in oxidized and reduced isoalloxazine ring systems, and found them to be similar. The ring current model derived for the oxidized isoalloxazine ring system is utilized for the phenoxazone ring system in this manuscript. The upfield ring current shifts in ppm in a plane at a distance of 3.4 Å from the phenoxazone ring and parallel to it.



(*ii*) *Ring Current Shifts.* The stacking geometries of the two GC Watson–Crick base pairs stacked on either side of the phenoxazone ring of the antibiotic in the 1 : 2 Act-D–d-pGpC complex as proposed by Sobell and Jain (Sobell and Jain, 1972) are presented in Figure 7. The CH_3-4 and CH_3-6 groups are predicted to be upfield shifted 0.2 ppm by the ring currents of the puring ring (Table VII) in the Sobell–Jain model of the complex. The experimental upfield shifts are 0.46 and 0.37 ppm for the CH_3 resonances (Table V). The nitrogen atom of the amino group at position 4 of the cytosine ring is stacked directly over each of the methyl groups. The anisotropy associated with the lone pair electrons of the hybridized nitrogen atom may account for the discrepancy between the observed ring current predicted results.

The predicted upfield shifts of the phenoxazone H-7 and H-8 from the ring currents of the G-C base pairs stacked on either side are 0.5 and 0.2 ppm, respectively (Table VII). Experimentally, upfield shifts of 0.68 and 0.23 ppm are observed for these benzenoid protons on complex formation (Table V).

For the guanosine ring which is stacked over the quinonoid ring, the H-8 proton lies directly over the oxygen atom of the

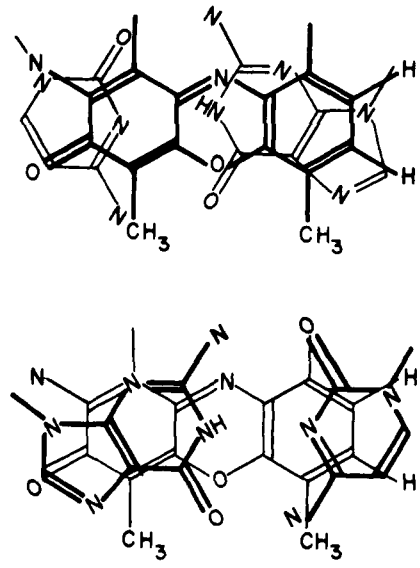


FIGURE 7: The overlap of a GC base pair on top of the phenoxazone ring and a CG base pair below the phenoxazone ring in the model of 1 : 2 Act-D–d-pGpC proposed by Sobell and Jain (1972).

phenoxazone ring carbonyl group. The magnetic anisotropy of the carbonyl group will contribute an 0.3-ppm upfield shift (Jackman and Sternhell, 1969). The average upfield shift on complex formation from the ring currents (0.1/2 ppm) and the anisotropy of the carbonyl group (0.3/2 ppm) of the phenoxazone ring is 0.2 ppm and is in agreement with the value observed experimentally (Table V).

Ts'o *et al.* (1969) have compared the cytosine H-5 and H-6 chemical shifts in pC and GpC. They found that the ring currents of the purine ring of GpC shift the cytosine H-5 resonance upfield by 0.4 ppm and H-6 resonance by 0.2 ppm. These contributions measured for the ribose analogs are assumed to remain unchanged in the deoxy analogs. The H-5 proton at 5.82 ppm in d-pGpC, pH 5.8, 26° (Table V), when corrected for the 0.4 ppm stacking contribution, results in a chemical shift of 6.22 ppm for the extended conformation. On complex formation this resonance shifts upfield by 0.15–6.07 ppm which compares favorably with a predicted 0.1-ppm upfield shift for the Sobell–Jain (1972) model of the complex

TABLE VII: Predicted Upfield Ring Current Shifts for the Sobell–Jain Model of the 1 : 2 Act-D–d-pGpC Complex.

	Ring Currents		
	Guanosine	Cytosine	Total
Phenoxazone			
CH_3-4	0.2	0	0.2
CH_3-6	0.2	0	0.2
H-7	0.3	0.2	0.5
H-8	0.1	0.2	0.2
	Ring Currents		
	Benzenoid	Quinonoid	Av
Guanosine			
H-8	0.1	0	0.05
Cytosine			
H-5	0.2	0	0.1
H-6	0.2	0	0.1

(Table VII). The same procedure leads to satisfactory agreement for the H-6 proton.

The guanosine and cytosine H-1′ protons are too distant from the phenoxazone ring to be affected by its ring current. Yet, 0.25-ppm chemical shifts in opposite directions are observed for the H-1′ protons of d-pGpC on addition of Act-D in aqueous solution (Table V). The H-1′ proton chemical shifts may be sensitive to the syn–anti nature of the glycosidic bond and the pucker of the sugar ring. Since the structure of d-pGpC in the free and complexed forms are quite different the experimental shifts may reflect such changes.

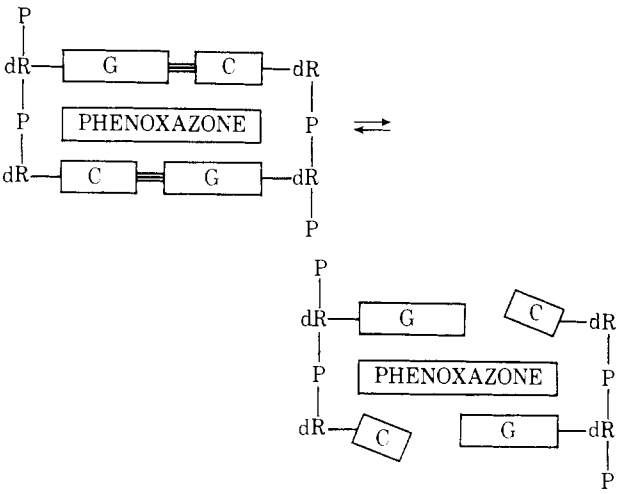
D. Hydrogen Bonding. (i) G–NH_2 Protons. The guanine amino protons (G–NH_2) are exposed to solvent water in the extended conformation(s) of d-pGpC in aqueous solution (Barry *et al.*, 1972). In the Sobell–Jain model of the complex, both amino protons participate in intermolecular hydrogen bonds. One of the exchangeable protons forms a Watson–Crick hydrogen bond with the cytosine carbonyl at position 1 while the second exchangeable proton forms a hydrogen bond with the carbonyl group of L-Thr on the pentapeptide lactone ring of Act-D.

Raszka and Kaplan (1973) have reported an 0.15-ppm downfield shift for the G–NH_2 protons of 0.2 M pG on complexation with an equimolar solution of pC in aqueous solution. The hexanucleotide d-$(pGpC)_3$ forms double-stranded Watson–Crick helical structures in aqueous solution. The NH_2 protons of guanosine and cytosine exhibit chemical shifts at 6.9 ppm and as far downfield as 8.6 ppm for the nonhydrogen bonded and Watson–Crick hydrogen bonded G–NH_2 protons, respectively (D. J. Patel, unpublished results). Thus, 0.15- (Raszka and Kaplan, 1973) and 1.7-ppm (D. J. Patel, unpublished results) downfield shifts are observed for the G–NH_2 protons on Watson–Crick base pairing with cytosine residue for the mononucleotides and complimentary hexanucleotides, respectively.

From the intercalation geometry of the Sobell–Jain model of 1:2 Act-D–d-pGpC (Sobell and Jain, 1972), and the phenoxazone upfield ring current contours, an average upfield shift of ~0.2 ppm is predicted for the G–NH_2 protons resulting from the ring currents of the phenoxazone ring. Experimentally, the G–NH_2 proton of d-pGpC undergoes a downfield shift (0.6 ppm at 30° and 0.45 ppm at 50°) on complexation with Act-D in aqueous solution (Figures 4 and 6), which persists over the temperature range 10–60° (Figure 6). It is assigned to the participation of the G–NH_2 proton in intermolecular hydrogen bonds with acceptor groups.

Temperature coefficients of $\geq 6 \times 10^{-3}$ ppm/deg for the exchangeable proton suggest exposure to solvent while coefficients of $\leq 2 \times 10^{-3}$ ppm/deg suggests its participation in a hydrogen bond or its burial from solvent water (Kopple *et al.*, 1969; for a review, see Bovey *et al.*, 1972).

The G–NH_2 protons of d-pGpC exhibit a temperature coefficient of 6.7×10^{-3} ppm/deg consistent with an extended conformation (Table VI, Figure 6). The G–NH_2 protons of d-pGpC in the 1:2 Act-D–d-pGpC complex exhibit a temperature coefficient of 18×10^{-3} ppm/deg (Table VI, Figure 6). This is a surprising result since hydrogen-bonded protons exhibit a temperature coefficient of $\leq 2 \times 10^{-3}$ ppm/deg. It is proposed that the stability of the Watson–Crick hydrogen bonds between GC residues in the complex is temperature dependent. At higher temperatures, there is rapid breakage and forming of these hydrogen bonds in the complex on the nmr time scale, such that the chemical shift is an average of the two states. Since the chemical shift of the nonhydrogen bonded state is upfield from the hydrogen-bonded form, increasing the temperature raises the population of the former state and results in an upfield shift. Indeed, the sample precipitates above 50° suggesting that the Act-D no longer complexes to d-pGpC and comes out of aqueous solution. A schematic of the suggested equilibrium is shown below.



(ii) Peptide Protons. The peptide protons of D-Val and L-Thr exhibit temperature coefficients of $\sim 3.5 \times 10^{-3}$ and 3.2×10^{-3} ppm, respectively (Table VI). In the Sobell–Jain model, the peptide protons of D-Val are hydrogen bonded to D-Val carbonyl groups on the other pentapeptide lactone ring while the L-Thr peptide protons are hydrogen bonded to the N-3 nitrogen of guanine. The temperature coefficient data are consistent with these residues participating in hydrogen bond formation in solution, too.

E. Summary of Conclusions. This research was initiated in an effort to apply high-resolution nmr spectroscopy to investigate the stacking, hydrogen bonding, and hydrophobic interactions responsible for the stability of peptide–nucleotide complexes.

Since nmr spectroscopy permits the observation of individual resonances, different aspects of the detailed stereochemical model of Sobell and Jain (1972) for the complex of Act-D with d-pGpC can be investigated in solution. Thus, the stoichiometry of the complex can be readily shown to be 1:2 Act-D–d-pGpC in solution by measurement of peak areas of identifiable resonances in the peptide and nucleotide.

This study has established that ^{31}P nmr spectroscopy can be a powerful probe for locating the intercalation site in a double-stranded nucleotide sequence since the internucleotide phosphates at the intercalation site exhibit downfield shifted ^{31}P resonances on complexation. It is not clear whether this property is specific for aromatic intercalating residues since the origin of the large downfield ^{31}P chemical shifts is not understood. The large range of ^{31}P chemical shifts and their sensitivity to intercalation permits an investigation of the peptide–nucleotide complexation kinetics from the temperature-dependent ^{31}P line widths.

Since complex formation involves stacking of groupings in the peptide and nucleotide, the chemical shifts of resonances predicted to arise from ring currents and magnetic anisotropies for a particular stereochemical model can be checked for magnitude and direction against experimental values in solution. The research of Krugh and Neely (1973) and this study have investigated the proton nmr spectra in the 1:2 Act-D–d-pGpC system and determined the proton complexation shifts to be consistent with the Sobell–Jain model (1972) of the stacking geometries in the complex.

In an effort to investigate the hydrogen bonding proposed for most models of peptide–nucleotide interactions, extensive investigations of the 1 : 2 Act-D–d-pGpC complex have been undertaken in H_2O solution. On complex formation, the guanine 2-amino (G–NH_2) protons undergo a large downfield shift consistent with hydrogen bond formation (Sobell and Jain, 1972).

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