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

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ABSTRACT: Complex formation between actinomycin D (Act-D) and the hexanucleotides d-ApTpGpCpApT and d-pGpCpGpCpGpC have been investigated by ^{1}H and ^{31}P nuclear magnetic resonance (nmr) spectroscopy. The ring NH (G-N_1H and T-N_3H) resonances in the 300-MHz proton nmr spectrum of d-ApTpGpCpApT in H_2O between 12 and 14 ppm have been assigned to specific base pairs in the known sequence from an analysis of the line widths and chemical shifts as a function of temperature. The T-N_3H resonances monitor the rapid fraying of the (AT) base pairs at the ends of the helix while the G-N_1H resonances of the $(GC)_{central}$ base pairs monitor the melting of the helix. From the gradual addition of Act-D to the deoxyhexanucleotides, the stoichiometry of the complex is determined to be 1:2 Act-D–hexanucleotide in solution with slow exchange on the nmr time scale of the antibiotic between the hexanucleotide and the complex. The complexation site is at the $(GC)_{central}$ base pairs since the G-N_1H resonances exhibit the largest chemical shift difference in the spectrum of 1:2 Act-D–d-ApTpGpCpApT in aqueous solution. Complex formation results in two large temperature-independent downfield ^{31}P chemical shifts consistent with intercalation of the Act-D between Watson–Crick base pairs. Thus, a combination of proton and phosphorus nmr studies support the Sobell–Jain proposal (*J. Mol. Biol. 68*, 21 (1972)) that the phenoxazone ring of Act-D intercalates between GC and CG Watson–Crick base pairs in double-helical d-ApTpGpCpApT in solution.

Actinomycin D (Act-D),[1] a peptide antibiotic, forms a tightly bound, noncovalent, reversible complex with double-helical DNA (for reviews, see Reich and Goldberg, 1964, Gale *et al.*, 1972, and Sobell, 1973). Several detailed stereochemical models have been proposed for the complex between Act-D and double-stranded DNA (Hamilton *et al.*, 1963; Muller and Crothers, 1968; Gursky, 1970; Sobell *et al.*, 1971). The Sobell–Jain model (Sobell and Jain, 1972) is based on an X-ray crystallographic analysis of the complex of Act-D with two deoxyguanosines (Jain and Sobell, 1972). Starting from this crystallographic data, Sobell and Jain (1972) proposed models for the 1:2 Act-D–d-pGpC and 1:2 Act-D–d-ApTpGpCpApT complexes. In this proposal, the phenoxazone ring intercalates between GC and CG Watson–Crick base pairs with the pentapeptide lactone rings extending over two base pairs on either side of the intercalation site. The symmetry axis relating the Act-D pentapeptide lactone rings coincides with the symmetry axis relating the sugar–phosphate backbone and base sequence of the double helix.

Complex formation between Act-D and deoxyhexanucleotides d-ApTpGpCpApT and d-pGpCpGpCpGpC in aqueous solution provide promising examples to investigate polypeptide–oligonucleotide interactions. The principles of intercalation, hydrogen-bonding, and hydrophobic interactions have been invoked for the recognition and complex stability of such macromolecular units (Sobell *et al.*, 1971; Adler *et al.*, 1972; Novak and Dohnal, 1973; Hélène *et al.*, 1971a,b; DeSantis *et al.*, 1973).

Experimental Section

Deoxynucleotides. d-ApTpGpCpApT (lot 421-54-A) and d-pGpCpGpCpGpC (lot 336-27-C) were purchased from Collaborative Research, Inc. The latter was passed through a Chelex column and lyophilized. ^{1}H nuclear magnetic resonance (nmr) spectra were run on 25-mg/ml solutions of these hexanucleotides. Actinomycin D was purchased from Merck and added as a solid to the deoxyoligonucleotide solutions.

Spectra. ^{1}H nmr spectra were run on a Varian HR 300-MHz frequency/field swept spectrometer. The variable-temperature probe maintained temperatures to $\pm 1°$. Spectra were time averaged to improve signal-to-noise ratio by using a Nicolet computer of average transients with 4096 channels. Chemical shifts are defined on δ scale in parts per million relative to sodium 3-(trimethylsilyl)propanesulfonate as standard. ^{31}P nmr spectra were run on an XL-100 spectrometer interfaced with a 20-K Nicolet 1080 computer (Sternlicht and Zuckerman, 1972). Spectra were recorded in the Fourier transform mode and referenced relative to external 16% phosphoric acid. pH was measured with an Ingold combination electrode and a Radiometer pH meter.

Results

In the all-or-none approximation, the transition from a fully Watson–Crick hydrogen-bonded double helix to separated single strands is referred to as the *melting* transition. For short oligonucleotide sequences, such as hexanucleotides, there is in addition a finite probability that the terminal base pairs are non-hydrogen bonded in the Watson–Crick double helix. This process is termed *fraying* at the ends of the double helix and occurs on a much faster time scale than the slower melting of the fully hydrogen-bonded double helix.

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

TABLE I: Proton Chemical Shift (ppm) and Line Widths (Hz, in parentheses) of Ring NH Resonances of Base Pairs in d-ApTpGpCpApT in Solution.

Conditions	Temp (°C)	Spectrum	$(AT)_{internal}$	$(AT)_{terminal}$	$(GC)_{central}$
H_2O, pH 7	3	1-I	13.77 (40)		12.69 (25)
	14		13.61 (180)		12.69 (32)
	25				~12.7 (~200)
0.1 M NaCl–H_2O, pH 7	0	1-II	13.77 (34)	~13.18 (100)	12.69 (25)
H_2O–MeOH, 3:2	0	1-III	13.73 (64)		12.74 (30)
H_2O–MeOH, 3:2	−11	1-IV	13.77 (52)	13.36 (56)	12.71 (52)

Proton Nmr Studies

Tritium-exchange studies have shown that the Watson–Crick hydrogen-bonded protons in a fully bonded double helix exhibit half-times for exchange with water on the order of minutes (Englander and von Hippel, 1972; Englander *et al.*, 1972). By contrast, the G-N_1H and T-N_3H resonances of the mononucleotides in aqueous solution cannot be detected due to rapid exchange with solvent suggesting exchange lifetimes of less than msec. These data suggest that the ring N protons in a fully hydrogen-bonded base pair can be observed in the high-resolution proton nmr spectrum. Increasing populations of the open form in rapid equilibrium with the Watson–Crick hydrogen-bonded form should result in broadening out of these resonances.

Earlier investigations (Kearns *et al.*, 1971) established that one exchangeable proton per base pair, namely the guanine N-1 proton (G-N_1H) and thymine N-3 proton (T-N_3H), is observed between 11 and 15 ppm downfield from the sulfonate standard in the high-resolution proton nmr spectra of tRNA in H_2O solution.

a. d-ApTpGpCpApT. The Watson–Crick double-helical form of the deoxyoligonucleotide d-ApTpGpCpApT exhibits a twofold symmetry axis such that only three base pairs need be considered and they are designated $(AT)_{terminal}$, $(AT)_{internal}$, and $(GC)_{central}$ (see structure **1**).

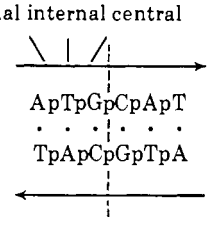


Figure 1-I presents the high resolution proton nmr spectrum of d-ApTpGpCpApT in H_2O between 11.5 to 14.5 ppm as a function of temperature. The earlier studies (Kearns *et al.*, 1971) predict the observation of three resonances, each of area two protons per double-stranded helix, corresponding to the $(AT)_{terminal}$, $(AT)_{internal}$, and $(GC)_{central}$ base pairs. At 3°, two distinct resonances at 13.77 and 12.69 ppm and a very broad resonance at ~13.15 ppm are observed. On raising the temperature, the resonance at 13.77 ppm broadens at 14° while the resonance at 12.69 ppm is broadened out at 25°. Since the ring NH resonance at ~13.15 ppm was very broad for the hexanucleotide in H_2O solution near the freezing point, an attempt was made to observe it under conditions where its area would be comparable to that of the ring NH resonances of the other base pairs. The spectrum of d-ApTpGpCpApT in 0.1 M NaCl–H_2O, pH 7 at 0° is presented in Figure 1-II. The resonances at 13.77 and ~13.15 ppm exhibit narrower line widths in the presence of salt. Three distinct resonances of equal area are observed in the high-resolution proton nmr spectrum of d-ApTpGpCpApT in H_2O:MeOH (3:2) at −11° between 12 and 14 ppm from the sulfonate standard (Figure 1-IV).

The *sequential* increase in line width for the three ring NH resonances observed in the spectrum of d-ApTpGpCpApT in H_2O–MeOH (3:2) and in H_2O between −11 and +25° reflect an increase in population of the nonhydrogen bonded form arising from the fraying and melting processes. The resonances have been assigned to the N protons of individual base pairs in double-stranded d-ApTpGpCpApT on the assumption that the exchange rate with solvent water increases on proceeding from the central base pairs to the terminal base pairs due to the fraying process. Thus, the assignments (Table I) of the resonances between 12 and 14 ppm in the spectrum of d-ApTpGpCpApT in H_2O at 3° (Figure 1-I) are presented as: T-N_3H, $(AT)_{terminal}$ ~13.15 ppm; T-N_3H, $(AT)_{internal}$ 13.77 ppm; G-N_1H, $(GC)_{central}$ 12.69 ppm.

b. 1:2 Act-D–d-ApTpGpCpApT. The proton nmr spectral changes on the gradual addition of Act-D to d-ApTpGpCpApT in H_2O, pH 7 at 0° are shown in Figure 2-I. On the addition of 0.5 equiv of the antibiotic (*i.e.*, one Act-D molecule per double stranded hexanucleotide), chemically shifted resonances are observed with the twofold symmetry of the oligonucleotide double helix removed in the spectrum of the complex. The Act-D lacks an exact twofold symmetry axis and therefore a tightly bound complex of Act-D and double-helical d-ApTpGpCpApT also lacks this element of symmetry. The addition of Act-D beyond 0.5 equiv resulted in no further spectral changes. The spectrum of d-ApTpGpCpApT on addition of 0.3 equiv of Act-D (*i.e.*, 0.6 Act-D/double-stranded hexanucleotide) is a superposition of the spectra of the hexanucleotide and the complex suggesting that the ex-

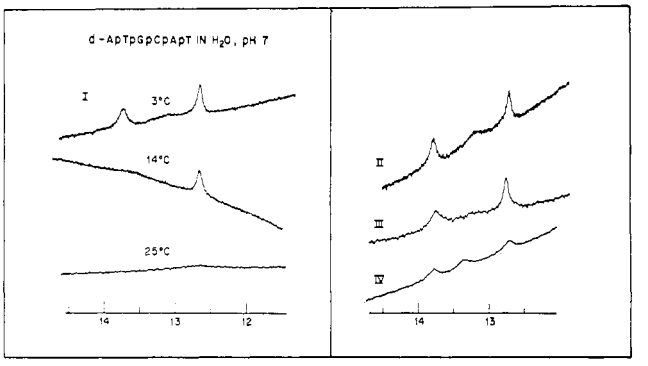


FIGURE 1: The temperature dependence of the high-resolution 300-MHz proton nmr spectrum of d-ApTpGpCpApT between 12 and 14 ppm downfield from standard sulfonate as a function of solvent: (I) H_2O, pH 7; (II) 0.1 M NaCl–H_2O, pH 7, 0°; (III) H_2O–MeOH, (3:2), 0°; (IV) H_2O–MeOH, (3:2), −11°.

TABLE II: Proton Chemical Shift (ppm) and Line Widths (Hz, in parentheses) for Ring NH Resonances of Base Pairs in 1:2 Act-D–d-ApTpGpCpApT in Solution.

Conditions	Temp (°C)	Spectrum	$(AT)_{internal}$	$(AT)_{terminal}$	$(GC)_{central}$
H_2O, pH 7	3	2-I	14.13 (54)		12.72 (24)
			13.95 (56)		12.31 (20)
	14		13.91		12.64 (18)
			13.80		12.27 (16)
	25	2-II	13.78 (88)		12.60 (17)
			13.57 (80)		12.27 (16)
H_2O–MeOH (3:2)	−21	2-III	14.55 (80)	13.33 (115)	12.87 (80)
			14.32 (80)		12.44 (80)
	−11	2-III	14.25 (66)	~12.9	12.79 (36)
			14.09 (70)		12.39 (36)

change of the antibiotic between double-helical hexanucleotides is slow on the nmr time scale ($\tau > 2$ msec) at 0°.

On complex formation, the G-N_1H resonances of the $(GC)_{central}$ base pairs in d-ApTpGpCpApT at 12.69 ppm are split by an 0.4-ppm chemical shift difference (Figure 2-I). One of the G-N_1H resonances remains unchanged while the chemical shift of the other is moved upfield by 0.4 ppm. The T-N_3H protons of the $(AT)_{internal}$ base pairs in d-ApTpGpCpApT at 13.77 ppm are shifted downfield by ~0.2 ppm into two resonances at 14.21 and 13.96 ppm in the complex (Figure 2-I). The T-N_3H protons of the $(AT)_{terminal}$ base pairs in d-ApTpGpCpApT were not detected in the spectrum of the complex at 0° (Figure 2-I). The proton nmr spectra of the 1:2 Act-D–d-ApTpGpCpApT complex in H_2O–MeOH (3:2) at −11 and −21° are presented in Figure 2-III. The T-N_3H resonance of the $(AT)_{terminal}$ base pairs can be detected at ~13.3 ppm at −21° and shifts upfield to ~12.9 ppm and broadens significantly at −11°. The chemical shifts are summarized in Table II.

The temperature dependence of the ring NH resonances of the 1:2 Act-D–d-ApTpGpCpApT complex in H_2O–MeOH (3:2), and in H_2O, are plotted in Figure 3. These resonances shift upfield with increasing temperature. The largest upfield shift is observed for the $(AT)_{terminal}$ base pair and the smallest upfield shift for the $(GC)_{central}$ base pair. The magnitude of the upfield shifts for the three base pairs parallels the linewidth changes observed (Figure 2). Thus, there is no linewidth change for the $(GC)_{central}$ base pairs and a significant broadening for the $(AT)_{internal}$ base pairs in the spectrum of the complex on raising the temperature to 25° (Figure 2-II). While the ring NH resonances of $(GC)_{central}$ and $(AT)_{internal}$ broaden out by 25° in the spectrum of d-ApTpGpCpApT in H_2O (Figure 1-I), they can still be observed at this temperature in the spectrum of the 1:2 Act-D–d-ApTpGpCpApT complex in H_2O (Figure 2-II).

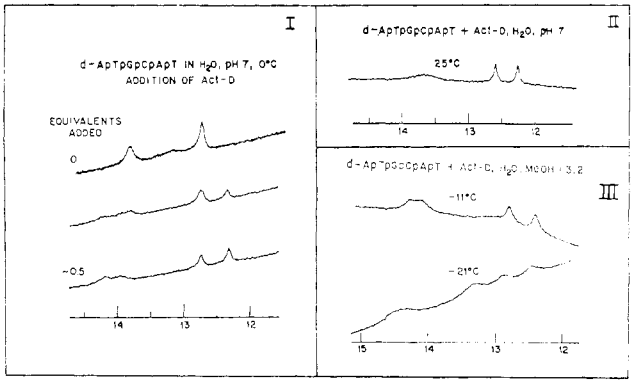
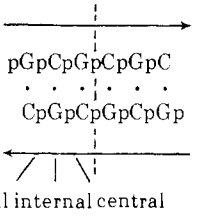


FIGURE 2: The high-resolution 300-MHz proton nmr spectra of d-ApTpGpCpApT in H_2O, pH 7, 0°, as a function of Act-D concentration (I). The high-resolution 300-MHz proton nmr spectra of 1:2 Act-D–d-ApTpGpCpApT in H_2O, pH 7, 25° (II); and in H_2O–MeOH (3:2) at −21 and −11° (III).

c. d-pGpCpGpCpGpC. Double-helical structures of d-(pGpC)$_3$ can be written which differ in the number of Watson–Crick hydrogen-bonding base pairs. Structure **2**, exhibiting the maximum Watson–Crick hydrogen bonds is assumed to predominate in aqueous solution. For structure **2**, there is an element of twofold symmetry and one need consider only three GC base pairs. The proton nmr spectra of d-(pGpC)$_3$



in 0.1 M NaCl–H_2O (pH 7) as a function of temperature are presented in Figure 4-I. Two resonances are observed at 13.3 and 13.2 ppm of approximately equal area. The G-N_1H resonances of the $(GC)_{terminal}$, $(GC)_{internal}$, and $(GC)_{central}$ base pairs are candidates for these two resonances. The resonances at 13.3 and 13.2 ppm are assigned to the G-N_1H protons of the nonterminal base pairs. On lowering the temperature to 0°, a very broad resonance at ~13.1 ppm is observed and assigned to the G-N_1H proton of the terminal base pair. On heating the sample above 7°, the G-N_1H resonances at 13.3 and 13.2 ppm broaden to the same extent as a function of temperature. Thus, the resonances at 13.3 and 13.2 ppm cannot be assigned to specific nonterminal (GC) base pairs (Figure 4-I).

d. 1:2 Act-D–d-pGpCpGpCpGpC. The proton nmr spectra on gradual addition of Act-D to d-(pGpC)$_3$ in 0.1 M NaCl–H_2O (pH 7, 7.5°) are presented in Figure 4-II. On addition of 0.5 equiv of antibiotic to d-(pGpC)$_3$ (*i.e.*, 1 Act-D per double-stranded hexanucleotide, a decrease in area at 13.2 to 13.3 ppm accompanies the observation of new resonances at 12.3 and 12.5 ppm. The spectrum after addition of 0.25 equiv of Act-D is a superposition of the spectrum in the absence and presence of 0.5 equiv of Act-D to d-(pGpC)$_3$ in H_2O solution (Figure 4-II).

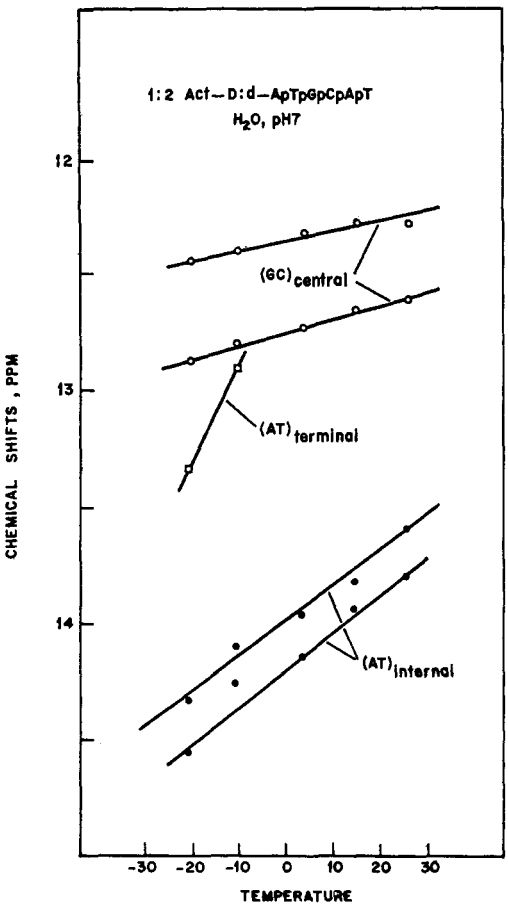


FIGURE 3: A plot of the T-N_3H and G-N_1H chemical shifts of the AT and GC base pairs in 1:2 Act-D–d-ApTpGpCpApT as a function of temperature. The data above 0° were accumulated in H_2O (pH 7) solution while the data below 0° were accumulated in H_2O–MeOH (3:2).

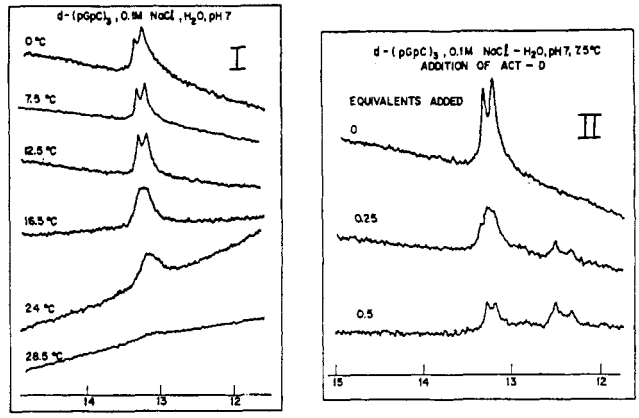


FIGURE 4: The high-resolution 300-MHz proton nmr spectra of d-(pGpC)$_3$ in 0.1 M NaCl–H_2O (pH 7) as a function of temperature between 12 and 14 ppm downfield from standard sulfonate (I). The high-resolution 300-MHz proton nmr spectra of d-(pGpC)$_3$ in 0.1 M NaCl–H_2O (pH 7) at 7.5° as a function of Act-D concentration (II).

B. Phosphorus Nmr Studies

a. d-ApTpGpCpApT. Double-stranded d-ApTpGpCpApT contains ten internucleotide phosphorus atoms with no terminal phosphate groups. This suggests that there should be five different ^{31}P nmr resonances with pH-independent chemical shifts. The 40-MHz ^{31}P nmr spectrum of d-ApTpGpCpApT in aqueous solution, referenced relative to external 16% phosphoric acid, exhibits several poorly resolved resonances between −1 and 0 ppm.

b. 1:2 Act-D–d-ApTpGpCpApT. The gradual addition of the antibiotic to 25 mg/ml of the hexanucleotide was investigated by ^{31}P nmr spectroscopy. On complex formation three phosphorus resonances are shifted downfield to new chemical shifts at 2.2, 1.25, and 0.55 ppm relative to external 16% phosphoric acid in D_2O. The chemical shifts of the remaining phosphorus atoms shift slightly on complex formation. Figure 5 presents the temperature dependence of the ^{31}P spectrum of >1:2 Act-D–d-ApTpGpCpApT (*i.e.*, greater than 1 equiv of antibiotic per double stranded hexamer). The downfield shifted resonances were observed at 10 and 30° with the ^{31}P resonance at 0.55 ppm (10°) shifting upfield to 0.2 ppm (30°).

c. d-pGpCpGpCpGpC. Double-stranded d-(pGpC)$_3$ contains ten internucleotide and two terminal phosphate atoms. The ^{31}P nmr spectrum of d-(pGpC)$_3$ in 0.1 M NaCl–0.01 M EDTA (8°) was recorded as a function of pH. Compared to the internucleotide phosphorus resonances between 0 and −1 ppm, a single resonance is observed to titrate between 1 and 4 ppm in the pH range 6–7. This titrateable resonance is assigned to the terminal phosphate of d-(pGpC)$_3$.

d. 1:2 Act-D–d-pGpCpGpCpGpC. The ^{31}P nmr spectrum of 1:2 Act-D–d-(pGpC)$_3$ in 0.1 M NaCl–0.01 M EDTA (8°) as a function of pH have been recorded in the region −1 to 6 ppm relative to external 16% phosphoric acid. The terminal phosphate can readily be identified since it exhibits a pH-dependent chemical shift. On complexation the spectral pattern between 0 and −1 ppm undergoes small chemical shift changes. At least four resonances are observed between 1 and 2 ppm in the complex and reflect downfield shifts on Act-D binding. These resonances exhibit an area of 1.5–2 phosphorus groups/double stranded hexanucleotide.

Discussion

A. G-N_1H and T-N_3H Resonances

The G-N_1H resonance of guanine and the T-N_3H resonance of thymine resonate at 10.9 and 10.0 ppm in dimethyl sulfoxide, respectively (Katz and Penman, 1966; Shoup *et al.*, 1966). They are not observed in H_2O due to rapid exchange with solvent. In a nucleic acid double helix these ring protons participate in Watson–Crick hydrogen bonds. Hydrogen-bond formation shifts the G-N_1H and T-N_3H resonances downfield and slows their exchange rate with water. Further, these ring NH resonances experience upfield ring current shifts from the purines of nearest neighbor base pairs, and are shielded from solvent by them. The chemical shift and line width of the ring NH resonances will depend on their location (*i.e.*, terminal *vs.* internal and nearest-neighbor sequence) and stability of the double helix (*i.e.*, fraying of the helix at its ends and on the concentration of double helix and single strands at a particular temperature).

a. Solvent Studies. The advantage of H_2O–MeOH (3:2) as a solvent lies in the fact that the proton nmr spectra can be recorded below 0° where H_2O freezes. Douzou and his colleagues have investigated macromolecules in mixed alcohol–water solvents at low temperatures (Travers *et al.*, 1970). Thus, on proceeding from 0° (Figure 1-III) to −11° (Figure 1-IV), the nmr spectra of d-ApTpGpCpApT in H_2O–MeOH (3:2) exhibit the T-N_3H proton of the $(AT)_{terminal}$ base pair at 13.36 ppm.

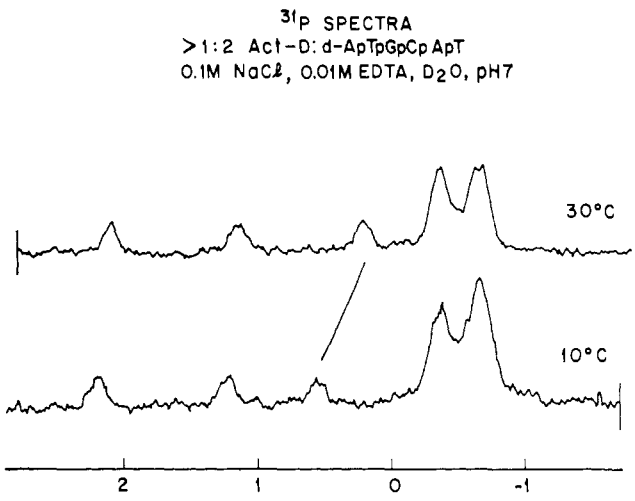


FIGURE 5: The 40-MHz ^{31}P nmr spectra of >1:2 Act-D–d-ApTpGpCpApT in 0.1 M NaCl–D_2O and 0.01 M EDTA (pH 7) at 30 and 10°.

Since the line widths reflect the stability of the Watson–Crick hydrogen bond, they are a measure of the fraying and melting properties of the double helix. The resonance at 13.75 ppm, assigned to the $(AT)_{internal}$ base pair in d-ApTpGpCpApT exhibits line widths of 40, 34, and 64 Hz in H_2O (pH 7, 0°), 0.1 M NaCl–H_2O (pH 7, 0°), and H_2O–MeOH (3:2) at 0°, respectively (Table I and Figure 1). This suggests that 0.1 M salt stabilizes while 40% methanol destabilizes the double helix of d-ApTpGpCpApT in aqueous solution as monitored at the $(AT)_{internal}$ base pair.

b. Temperature Studies. The observed average chemical shift of the ring NH of each Watson–Crick base pair is the weighted average of the populations of double-helical and open forms. Increasing populations of the open form in this equilibrium will shift the resonance upfield and broaden it. Thus, the T-N_3H of the $(AT)_{terminal}$ base pair shifts from 13.36 ppm in H_2O–MeOH (3:2) at −11° to ~13.18 ppm in 0.1 M NaCl–H_2O at 0° (Table I), suggesting that the population of open form for this base pair has increased over this temperature range. Since this proton shifts upfield as an average resonance, the exchange process for fraying is fast on the nmr time scale. The chemical shift difference, $\Delta\nu$, between double-helical (~14 ppm) and open (~10 ppm) forms of T-N_3H is ~4 ppm and therefore the average lifetime of exchange is $\tau < \pi\sqrt{2\Delta\nu})^{-1}$, and is computed to be <0.2 msec. The chemical shifts of the T-N_3H resonance of the $(AT)_{internal}$ and the G-N_1H resonance of the $(GC)_{central}$ base pairs remain unchanged at 13.77 and 12.7 ppm, respectively, in this temperature range (Table I). This suggests that the nonterminal base pairs of the double helix are stable over this temperature range (−11 to 0°) while there is fraying at the ends of the helix. On raising the temperature from 3 to 14°, the chemical shift of the G-N_1H proton of the $(GC)_{central}$ base pair remains unchanged at 12.69 ppm while the chemical shift of the T-N_3H of the $(AT)_{internal}$ base pair shifts upfield from 13.77 to 13.61 ppm and broadens considerably. Thus, though the $(GC)_{central}$ base pairs of the hexanucleotide double helix are stable, the fraying extends to all the (AT) base pairs in the temperature range 3–14°. On raising the temperature from 14 to 25°, the G-N_1H protons of the $(GC)_{central}$ base pairs broaden out, presumably due to the onset of the melting of the hexanucleotide double helix (Figure 1-I).

The two resonances at 13.3 and 13.2 ppm in the high-resolution proton nmr spectrum of d-$(pGpC)_3$ in 0.1 M NaCl–H_2O, pH 7 (Figure 4), have been assigned to the G-N_1H protons of the nonterminal GC base pairs of the double-stranded structure of d-$(pGpC)_3$ exhibiting six intramolecular hydrogen bonds (structure **2**). Since the chemical shift and line-width changes with temperature are similar for the $(GC)_{internal}$ and $(GC)_{central}$ base pairs, it is suggested that the broadening process observed for these resonances monitors the onset of the double-helix melting to the single-strand structures. On lowering the temperature to 0°, a broad resonance is observed at ~13.1 ppm, and may be assigned to the G-N_1H resonance of the terminal GC base pairs. The data suggest that double-stranded d-$(pGpC)_3$ in 0.1 M NaCl–H_2O exhibits fraying of the terminal base pairs only.

The temperature dependence of the chemical shifts of the ring NH resonances in the 1:2 Act-D–d-ApTpGpCpApT in H_2O, pH 7 between 0 and 25° and in H_2O–MeOH (3:2) between −20 and −10° are plotted in Figure 3. The ring G-N_1H protons of the $(GC)_{central}$ base pairs exhibit the lowest temperature dependence of the chemical shift, while the T-N_3H of the $(AT)_{terminal}$ base pairs exhibit the highest temperature dependence of the chemical shift. Even though there are significant[2] proportions of the open form in rapid equilibrium with the Watson–Crick hydrogen-bonded form of the $(AT)_{internal}$ base pairs, the T-N_3H resonance can be observed up to 25°. This suggests that the antibiotic pentapeptide lactone rings may shield the T-N_3H protons of the $(AT)_{internal}$ base pairs in the open form from solvent H_2O in the complex, thus lowering the exchange rate with solvent H_2O. The Sobell–Jain model (1972) proposes that the pentapeptide lactone rings extend two base pairs on either side of the intercalation site and this would encompass the $(GC)_{central}$ and $(AT)_{internal}$ Watson–Crick base pairs.

c. Complex Formation. The ring NH resonances of the $(GC)_{central}$ and $(AT)_{internal}$ base pairs for d-ApTpGpCpApT can be followed on gradual addition of Act-D in Figure 2-I. Since these resonances have been identified with specific base pairs in the double-stranded hexanucleotide, they can also be readily identified in the complex (Table II). On complex formation the largest nonequivalence, 0.4 ppm, is observed experimentally for the G-N_1H protons of the $(GC)_{central}$ base pairs and the smallest nonequivalence is observed experimentally for the G-N_1H protons of the $(GC)_{central}$ base pairs and the smallest nonequivalence is observed experimentally for the T-N_3H protons of the $(AT)_{terminal}$ base pairs. The data strongly suggest that the asymmetrically substituted phenoxazone ring of Act-D binds at the $(GC)_{central}$ Watson–Crick base pairs in double-helical d-ApTpGpCpApT.

The Sobell and Jain (1972) proposed intercalation of the antibiotic between GpC sequences in d-ApTpGpCpApT. In this intercalation model, the G-N_1H resonance of one GC base pair lies over the bezenoid ring and the G-N_1H resonance of the other GC base pair lies over the quinonoid ring of the phenoxazone. The experimental nonequivalence of the G-N_1H resonances of the GC base pairs in the complex could result from the different magnitudes of the upfield ring current contribution from the phenoxazone ring.

It has been proposed (Sobell, 1973) that the specificity of Act-D complexation to DNA double helices (Reich and Goldberg, 1964) and lack of complexation to RNA and DNA–RNA double helices (Reich, 1964; Haselkorn, 1964; Gellert *et al.*, 1965) indicates a preference for the B rather than the

[2] Since the total shift difference between open and Watson–Crick hydrogen-bonded forms is ~4 ppm, the 1-ppm upfield shift between −30 and +30° suggests a 25% increase in the open form.

A form of double-helical polyribonucleotides. Since the nmr studies have demonstrated complex formation between Act-D and d-ApTpGpCpApT in aqueous solution, double-helical d-ApTpGpCpApT in H_2O exists in the DNA-B form at the concentrations (25 mg of single strand/1 ml) utilized in this study.

B. Nucleotide Phosphate Groups

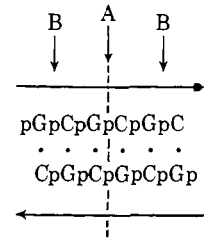
Prior to discussing the ^{31}P nmr data on the double-stranded hexanucleotides and their complexes with Act-D, it is necessary to consider data on the dinucleotide d-pGpC. d-pGpC contains two phosphorus atoms and the ^{31}P nmr spectrum in D_2O contains two resonances. These resonances can be readily assigned from a pH study since the terminal phosphate shifts ~3 ppm downfield on changing the pH from 5 to 8 while the chemical shift of the internucleotide phosphate is unaffected. The Sobell–Jain model (1972) of the 1:2 Act-D–d-pGpC complex proposes intercalation of the phenoxazone ring between GC and CG base pairs of the Watson–Crick antiparallel double-helical arrangement of two molecules of the dinucleotide. The ^{31}P chemical shift of the terminal phosphate of d-pGpC is unchanged on complexation with Act-D while the internucleotide phosphorus chemical shift moves 1.6 ppm downfield in the 1:2 Act-D–d-pGpC complex. Thus, the intercalation of the antibiotic between two Watson–Crick base pairs is reflected in the large ^{31}P downfield shift for the internucleotide phosphates of the same base pairs.

Complex formation of double-stranded d-ApTpGpCpApT with Act-D can be followed by ^{31}P nmr spectroscopy. In the >1:2 Act-D–d-ApTpGpCpApT complex in H_2O pH 7, 10°, three new ^{31}P resonances are observed at 2.2, 1.2, and 0.55 ppm (Figure 5). These resonances exhibit an area of 1 phosphorus each to approximately 7 phosphorus resonances between 0 and −1 ppm. The temperature-independent lines at 1.2 and 2.2 ppm are assigned to the internucleotide phosphate atoms at the intercalating site. Since the Sobell–Jain model has proposed intercalation between GpC sequences in d-ApTpGpCpApT and the proton nmr data support this proposal, the downfield shifted resonances at 1.2 and 2.2 ppm are assigned to the internucleotide GpC phosphate groups. There is slow exchange of Act-D between free and complexed d-ApGpCpApApT indicating a tightly bound complex. One of the GpC phosphates faces the nonpolar benzenoid ring of the phenoxazone while the GpC phosphate on the other chain faces the polar quinonoid ring of the phenoxazone. A hydrogen bond through a water molecule has been proposed for the 2-amino group of the phenoxazone ring and its neighboring phosphate group. The different environments for the GpC phosphates on the two chains of the double-stranded hexanucleotide in the complex due to asymmetry of the intercalating phenoxazone ring could account for the chemical shifts at 2.2 and 1.2 ppm. The ^{31}P resonance at 0.55 ppm (10°) and 0.2 ppm (30°) in the spectrum of >1:2 Act-D–d-ApTpGpCpApT cannot be readily assigned (Figure 5) since there should be only one intercalation binding site per hexanucleotide. The presence of a third downfield resonance could arise from weak surface binding. The remaining internucleotide phosphates show small changes between 0 and −1 ppm on complex formation. Since the helix undergoes unwinding on intercalation, these small shifts are not unexpected and reflect small perturbations as monitored by ^{31}P nmr spectroscopy at groups distant from the intercalation site.

Downfield shifts are also observed between 1 and 2 ppm in the ^{31}P spectrum of 1:2 Act-D–d-pGpCpGpCpGpC in aqueous solution. These resonances can be readily separated from the terminal phosphate resonances since the latter exhibit pH-dependent chemical shifts. The downfield shifted resonances exhibit two narrow ^{31}P signals which are slightly downfield from two broad ^{31}P signals. Since there are two binding sites (A and B) on double-stranded d-pGpCpGpC-pGpC, each site results in two downfield ^{31}P resonances. They appear to be approximately equally populated since the four downfield resonances between 1 and 2 ppm exhibit similar areas.

TABLE III: Proton Chemical Shifts (ppm) of the G-N_1H Resonance of GC Base Pairs.

Sample	Conditions	Temp (°C)	Chemical Shifts
d-$(pGpC)_3$	0.1 M NaCl–H_2O, pH 7	0	13.35, 13.25
		7.5	13.31, 13.21
		12.5	13.30, 13.19
		16.5	13.27, 13.19
		24	13.16
1:2 Act-D–d-$(pGpC)_3$	0.1 M NaCl–H_2O, pH 7	0	13.27, 13.19, 12.51, 12.32



Summary

Complex formation between Act-D and the hexanucleotides d-ApTpGpCpApT and d-pGpCpGpCpGpC have been investigated by ^1H and ^{31}P nmr spectroscopy. The proton spectra monitor the Watson–Crick base pairs (G-N_1H and T-N_3H resonances) while the phosphorus spectra monitor the sugar–phosphate backbone.

A double-stranded antiparallel helical structure (1) is assigned to the hexanucleotide d-ApTpGpCpApT since resonances attributed to the G-N_1H proton in GC base pairs and T-N_3H proton in AT base pairs are observed between 12 and 14 ppm in aqueous solution. The resonances have been assigned to specific base pairs in the known sequence from an analysis of the temperature dependence of the line widths and chemical shifts of the ring NH resonances. The assignments are made assuming that the exchange rate with water is more rapid for the terminal base pairs as compared to the central base pairs. Raising the temperature of the hexanucleotide solution has two effects on the stability of the fully hydrogen-bonded double-helical structure. The melting process, which involves the conversion of the double-stranded structure to separate single strands, can be monitored by the G-N_1H resonances of the $(GC)_{central}$ base pairs. Rapid fraying of the ends of the double-stranded structure can be monitored by the T-N_3H resonances of the $(AT)_{terminal}$ base pairs between −11 and 0° and the $(AT)_{internal}$ base pairs between 0 and 14°. In all cases, upfield shifts and line broadening reflects increasing popula-

tions of the non-hydrogen-bonded forms. The fraying process is fast on the nmr time scale since the ring NH resonances shift as average signals giving a lifetime $\tau < 0.2$ msec. Since the hexanucleotides d-ApTpGpCpApT and d-pGpCpGpCpGpC bind Act-D in solution, these double-helical structures are in the DNA-B form in solution.

From the gradual addition of Act-D to the deoxyhexanucleotides, the stoichiometry of the complex has been determined to be 1:2 Act-D–hexanucleotide in solution. At low Act-D concentrations, exchange of the antibiotic between the hexanucleotide and the complex is slow on the nmr time scale. An analysis of the ^1H and ^{31}P spectra yields a lifetime, $\tau > 2$ msec for the complex in aqueous solution. Complex stability increases in the order 1:2 Act-D–d-pG < 1:2 Act-D–d-pGpC < 1:2 Act-D–d-ApTpGpCpApT.

The 1:2 Act-D–d-ApTpGpCpApT complex melts at a higher temperature than double-stranded d-ApTpGpCpApT since the G-N_1H resonances of the $(GC)_{internal}$ base pairs can be observed in the spectrum of the complex at 25° while they are broadened out at this temperature in the spectrum of the hexanucleotide.

The phenoxazone ring of Act-D is asymmetrically substituted and complex formation with the hexanucleotides d-ApTpGpCpApT and d-pGpCpGpCpGpC results in the removal of the exact twofold symmetry of the double helix. The largest chemical shift difference has been observed for the G-N_1H resonances of the $(GC)_{central}$ base pairs on complexation, while the T-N_3H resonances of the $(AT)_{internal}$ base pairs reflect a smaller chemical shift difference and the $(AT)_{terminal}$ base pairs none at all. This suggests that the complexation site is at the (GC) base pairs. Further, complex formation results in two large temperature-independent (10–30°) downfield ^{31}P chemical shifts. These downfield shifts are assigned to the ^{31}P resonances of the internucleotide phosphate groups at the intercalation site. (Model system studies have shown that the complex of Act-D with d-pG and d-pCpG which results in base stacking does not shift the ^{31}P resonances of the phosphates while complex formation of Act-D with d-pGpC, which results in intercalation, downfield shifts the internucleotide phosphorus resonances by 1.6 ppm.) Thus, a combination of proton and phosphorus nmr studies suggest that the phenoxazone ring of Act-D intercalates between GC and CG base pairs in double-helical d-ApTpGpCpApT.

The temperature dependence of the chemical shifts of the ring NH resonances in 1:2 Act-D–d-ApTpGpCpApT suggest fraying at the (AT) base pairs which is fast on the nmr time scale. The fraying is greatest at the $(AT)_{terminal}$ base pairs and significant at the $(AT)_{internal}$ base pairs. Moreover, the pentapeptide lactone rings shield the ring N protons of the nonterminal base pairs from solvent water.

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