Phosphorus-31 Nuclear Magnetic Resonance Studies of Actinomycin D, Ethidium Bromide, and 9-Aminoacridine Complexes with Dinucleotides†

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ABSTRACT: Phosphorus-31 nuclear magnetic resonance spectra of actinomycin D, ethidium bromide, and 9-aminoacridine complexes with deoxydinucleotides and ribodinucleoside monophosphates are reported. In the 2:1 pdGpdC-actinomycin D complex, the internucleotide phosphorus resonances exhibit individual resonances in the slow exchange region at −18 °C which are −1.7 and −2.4 ppm downfield of the resonance from the internucleotide phosphorus (atom) in a pdGpdC solution under similar experimental conditions. These complexation shifts result from the formation of a miniature intercalated complex. The formation of an intercalated complex of actinomycin D with the complementary mixture of deoxydinucleotides pdGpdT and pdApdC resulted in complexation shifts of −0.2 and −0.75 ppm (−16 °C) for the internucleotide phosphates of pdGpdT and pdApdC, respectively. The phosphorus-31 complexation shifts are also reported for several other actinomycin D solutions with mixtures of complementary and noncomplementary deoxydinucleotides. Ethidium bromide forms miniature intercalated complexes with pdCpdG and CpG which result in complexation shifts of −0.1 ppm and +0.2 ppm (2:1 nucleotide-ethidium bromide solutions at 6 °C), respectively. A +0.15 ppm complexation shift is observed in a 2.2:1 CpG-9-aminoacridine solution at 3 °C. These phosphorus-31 chemical shift data suggest that both the structure of the intercalating drug and the sequence of the nucleotides at the intercalation site influence the geometry of the intercalated complex, although at the present time it is not possible to quantitatively interpret these complexation shifts in terms of changes in the geometry of the sugar-phosphate backbone of the nucleic acid.

Phosphorus-31 nuclear magnetic resonance has been used to study a variety of systems, including the investigation of the structure of nucleic acids (e.g., Mandel and Westley, 1964; Gueron, 1971; Blumenstein and Raftery, 1972; Weiner et al., 1974; Gueron and Shulman, 1975; Feeney et al., 1975, and Patel and Canuel, 1976), and drug-nucleic acid complexes (Patel, 1974a,b, 1976). In general, the ^{31}P chemical shifts have been shown to be dependent upon the conformation of the nucleic acids. For example, Patel (1974) observed that the chemical shift of the internucleotide phosphate of the deoxydinucleotide pdG*p*dC moved downfield upon complex formation with actinomycin D under conditions where actinomycin D binds two pdGpdC molecules to form a miniature intercalated complex (e.g., see Krugh and Neely, 1973b). This initial experiment as well as related phosphorus-31 studies of actinomycin D binding to dGpdC sequences of tetra- and hexanucleotides suggested that phosphorus-31 spectroscopy may provide useful information on the conformation of the nucleic acid backbone in drug-nucleic acid complexes. Gorenstein (1975) and Gorenstein and Kar (1975) have performed CNDO calculations which indicate that the ^{31}P chemical shifts of nucleic acids are largely dependent upon the bond angles and torsional angles of the backbone. The present experiments were undertaken to determine the general utility of phosphorus-31 spectroscopy in the study of drug-nucleic acid complexes, in the hope of providing a broader experimental basis to interpret changes in the phosphorus-31 chemical shifts of nucleic acids. In this manuscript, we present the ^{31}P data for the complexation of the intercalating drugs actinomycin D, ethidium bromide, and 9-aminoacridine with both deoxydinucleotides and ribodinucleoside monophosphates. It is important for the reader to be aware of our detailed spectroscopic studies on the complexes of actinomycin D and ethidium bromide with the deoxynucleotides and ribodinucleoside monophosphates where we have been able to characterize the geometries of the complexes, since in this manuscript we will take advantage of this information (e.g., see Krugh et al., 1977; Chiao and Krugh, 1977; Krugh and Reinhardt, 1975; Krugh et al., 1975; Krugh and Chen, 1975).

Experimental Procedures

Ethidium bromide, 9-aminoacridine, and the ribodinucleoside monophosphate CpG were purchased from Sigma Chemical Co. Actinomycin D was purchased from Merck, Sharp, and Dohme Co. The deoxydinucleotides were purchased from Collaborative Research Corp. Ethidium bromide and actinomycin D were used without further purification. 9-Aminoacridine, which was twice recrystallized from acetone, gave a single spot when analyzed by paper chromatography. Trace paramagnetic impurities contained in the deoxydinucleotides were removed by treatment with Chelex-100 (Bio-Rad), followed by filtration and lyophilization of the dinucleotides prior to use. The dinucleotide solutions were prepared in either a D_2O or in a 30% methanol-d_4/D_2O mixture. Disodium ethylenediaminetetraacetate ($\sim 10^{-4}$ M) was added to both solvents. The pH was adjusted with NaOD and DCl to a meter reading of 7.00 ± 0.05 for the D_2O solutions and 7.5 ± 0.2 for the methanol-d_4/D_2O solutions. The dinucleotide concentrations were determined spectrophotometrically using the extinction coefficients listed in P-L Biochemicals catalog No. 104 with the exception of pdA-dC (ϵ_{262} was found to be

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TABLE I: Phosphorus-31 Chemical Shifts of the Internucleotide Phosphate Resonance.[a]

C*p*G	−0.10
pdC*p*dC	−0.24
pdC*p*dG	−0.31
pdG*p*dC	−0.35
pdA*p*dC	−0.36
pdC*p*dA	−0.40
pdG*p*dG	−0.44
pdG*p*dT	−0.54
pdG*p*dA	−0.60

[a] Chemical shifts are in ppm relative to 15% H_3PO_4 with an estimated error of ±0.02 ppm. Negative values occur upfield of the external reference. All solutions were prepared in D_2O; pH meter = 7.0 ± 0.05; T = 25 ± 3 °C.

22.2×10^3 at pH 7.0). Both actinomycin D and 9-aminoacridine were added directly to the dinucleotide solutions by weight, while the ethidium bromide was added by small volumes from a concentrated stock solution.

The ^{31}P NMR spectra were recorded on a JEOL PFT-100 Fourier transform spectrometer operating at 40.48 MHz interfaced with a JEOL EC-100 computer for data accumulation. Deuterium was used as an internal lock, and proton noise decoupling was used throughout. The sample temperature was regulated with a JEOL VT-3 temperature controller; the temperature was measured before and after each experiment. Sample solutions of 1 mL were prepared in 10-mm NMR tubes fitted with Teflon plugs to prevent vortexing. An aqueous tetrahydroxyphosphonium perchlorate solution, $P(OH)_4^+ ClO_4^-$ (prepared by mixing H_3PO_4 with $HClO_4$; Glonek and van Wazer, 1974) was sealed in a 1-mm capillary and was used as a secondary external reference by inserting the capillary through a hole in the Teflon plug. Since the chemical shift of the $P(OH)_4^+$ reference was sensitive to temperature changes and to magnetic susceptibility effects of the glass capillary, each $P(OH)_4^+$ capillary was also measured relative to an external 15% H_3PO_4 solution at room temperature (25 ± 3 °C). The individual nucleotide chemical shifts were also found to be dependent upon temperature as well as solvent effects. Therefore, it was necessary to carefully measure the nucleotide resonances relative to $P(OH)_4^+$ in the absence and presence of a given drug using the same $P(OH)_4^+$ reference capillary under identical experimental conditions of temperature, solvent, etc. Complexation shifts upon binding were determined using the relationship: $\Delta\delta = \delta$(nucleotide solution) − δ(nucleotide-drug solution), where a negative $\Delta\delta$ indicates that the resonance moved downfield upon the addition of the drug. The ^{31}P chemical shifts of the dinucleotides were also measured at room temperature relative to an external 15% H_3PO_4 standard. All ^{31}P NMR spectra were measured using 60° pulses (to minimize saturation effects) with a repetition rate of 2.3 s. A spectral width of 500 Hz with 2K data points was used for accumulation, which provides a maximum resolution of 0.48 Hz (0.01 ppm).

Results

Dinucleotides. The phosphorus-31 chemical shifts of the phosphorus atom in the 3′–5′ phosphodiester linkage (i.e., the internucleotide phosphates) of several dinucleotides are presented in Table I. The deoxydinucleotides also contain a 5′-terminal phosphate (monoester) whose resonance is ~3 ppm downfield from 15% H_3PO_4 at pH 7. The chemical shifts of the internucleotide phosphates were found to be essentially independent of pH in the range pH 5 to 10, while the terminal phosphate resonances shifted approximately 3.5 ppm over this pH range. Thus, the pH dependence of the terminal phosphate provides a basis for assigning the ^{31}P deoxydinucleotide resonances (Crutchfield et al., 1967; Patel, 1974a). In the absence of paramagnetic impurities, both the terminal and internucleotide phosphate resonances appear as relatively narrow resonances. Although the chemical shifts of the internucleotide phosphate resonances of the deoxydinucleotide sequence isomers (e.g., pdC*p*dG and pdG*p*dC) are approximately the same (Table I), the base composition does influence the observed chemical shifts. In the concentration range of the present experiments (4–10 mM dinucleotide), and in the absence of the drugs, the dinucleotides exist predominantly in the single-stranded form with little dimerization of the self-complementary dinucleotides to form miniature double helices (e.g., see Krugh and Young, 1975; Young and Krugh, 1975; Krugh et al., 1976). A comparison of the internucleotide phosphate chemical shift of 4 mM pdG*p*dC (−0.35 ppm; Table I) to that of 50 mM pdG*p*dC (−0.48 ppm; Patel, 1974a) may reflect such structural changes, although these chemical-shift differences are too small to deserve much significance without further documentation.

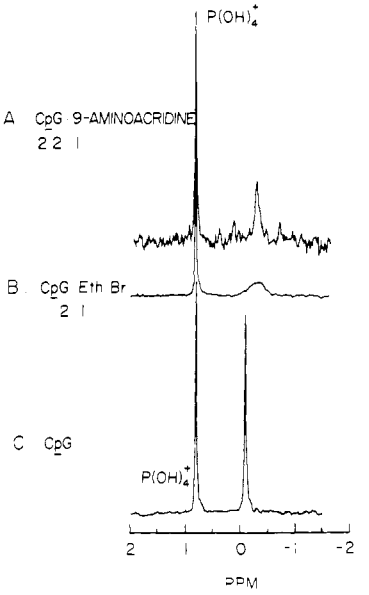

FIGURE 1: 40.48 MHz ^{31}P Fourier transform NMR spectra of solutions of (A) 5.7 mM CpG with 2.6 mM 9-aminoacridine at 3 °C; (B) 8 mM CpG with 4 mM ethidium bromide at 6 °C; and (C) 9.8 mM CpG at 6 °C. The $P(OH)_4^+$ resonance (external capillary) is located 0.80-ppm downfield from 15% H_3PO_4 in these spectra. EthBr, ethidium bromide.

Complexes of Ethidium Bromide and 9-Aminoacridine with CpG. Our previous fluorescence, circular dichroism, and proton magnetic resonance experiments have shown that ethidium bromide will form a miniature intercalated complex with two CpG molecules (Krugh et al., 1975; Krugh and Reinhardt, 1975), which has recently been confirmed by Davanloo and Crothers (1976). In these same experiments, we also observed that ethidium preferentially binds to pyrimidine-purine sequence dinucleotides (e.g., CpG, pdCpdG, pdTpdA, etc.) when compared to the purine-pyrimidine sequence dinucleotides (e.g., GpC, pdGpdC, pdApdT, etc.). The phosphorus-31 spectra of CpG, a CpG-ethidium bromide solution, and a CpG-9-aminoacridine solution are shown in Figure 1. The changes in the chemical shifts of the internucleotide phosphate resonances that result from complex for-

TABLE II: Complexation Shifts ($\Delta\delta$) of the Internucleotide Phosphate Resonances for Ethidium and 9-Aminoacridine Complexes.

	Nucleotide/drug	T (°C)	$\Delta\delta^a$ (ppm)
pdCpdG + ethidium bromide	2:1	26	−0.08
	2:1	6	−0.08
	1:1	6	−0.14
	0.67:1	6	−0.14
CpG + ethidium bromide	2:1	28	+0.17
	2:1	6	+0.21
CpG + 9-aminoacridine	2·2:1	24	+0.05
	2·2:1	3	+0.15

[a] Complexation shifts, $\Delta\delta$(ppm), are determined at the indicated temperatures using $\Delta\delta = \delta$(nucleotide solution) − δ(nucleotide-drug solution) under the conditions listed in the table. Negative values indicate downfield complexation shifts.

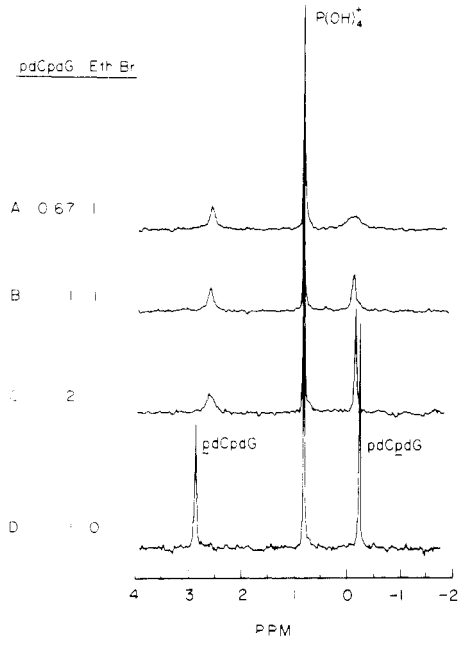

FIGURE 2: 40.48 MHz ^{31}P Fourier transform NMR spectra of pdCpdG and pdCpdG-ethidium bromide solutions at 6 °C. The concentrations of pdCpdG were: (A) 4.8; (B) 5.4; (C) 6.1; and (D) 7.1 mM. The ethidium bromide concentration is given as the ratio of the pdCpdG concentration to the ethidium bromide concentration. The $P(OH)_4^+$ external capillary resonance is 0.75-ppm downfield from 15% H_3PO_4.

mation (i.e., the complexation shifts, $\Delta\delta$) are listed in Table II. As can be seen from Figure 1 and Table II, both ethidium bromide and 9-aminoacridine induce upfield shifts (+0.21 and +0.15 ppm, respectively) of the internucleotide phosphate resonance of CpG in the 2:1 nucleotide-drug complexes at low temperature. There is also a reduction in the integrated intensities of these peaks as compared to an equivalent CpG solution at the same concentration. Raising the temperature of these solutions to ambient probe temperature (24–28 °C) results in a narrowing of the phosphorus-31 line widths and a decrease in the complexation shifts, especially for the 2:1 CpG–9-aminoacridine solution (Table II).

Ethidium Bromide Complexes with pdCpdG. The ^{31}P spectra of pdCpdG and a series of pdCpdG solutions with ethidium bromide at 6 °C are presented in Figure 2. The corresponding complexation shifts ($\Delta\delta$) upon binding are listed in Table II. For a 2:1 pdCpdG–ethidium bromide solution, we observe a small downfield complexation shift ($\Delta\delta = -0.08$ ppm) of the internucleotide phosphate resonance and an upfield shift ($\Delta\delta \simeq +0.2$ ppm) of the terminal phosphate resonance at both 26 and 6 °C. At lower dinucleotide–ethidium bromide ratios, the internucleotide phosphate resonance moves further downfield and is progressively broadened (Figure 2). Both the terminal and internucleotide phosphate resonances of pdCpdG show a decrease in their integrated intensities upon complexation with ethidium bromide when compared to the intensities of an equivalent pdCpdG solution.

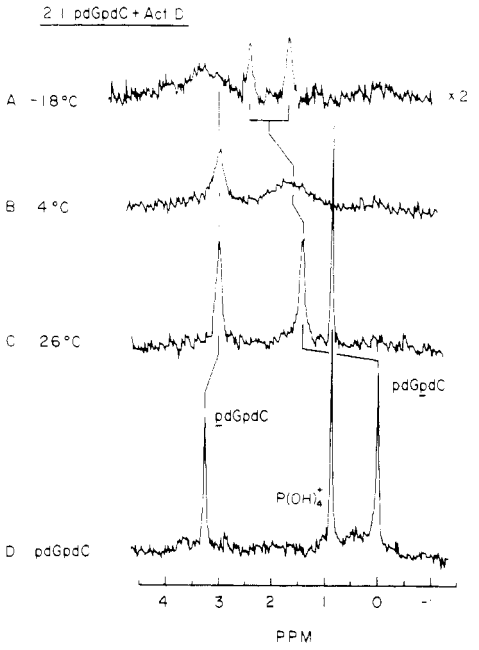

FIGURE 3: 40.48 MHz ^{31}P Fourier transform NMR spectra of 2:1 pdGpdC–actinomycin D solutions as a function of temperature. The concentrations of pdGpdC were (A) 5 mM in 30% (v/v) methanol-d_4/D_2O solvent; (B–D) 10.8 mM in D_2O. All chemical shifts are refereneed relative to the chemical shift of the internucleotide phosphate of a pdG*p*dC solution recorded in the same solvent and under the same experimental conditions used to measure the spectra of the 2:1 pdGpdC:actinomycin D solutions shown above. When measured relative to 15% H_3PO_4, the free pdG*p*dC chemical shifts are −0.98, −0.38, and −0.35 ppm in A, B, and C, respectively. Act D, actinomycin D.

Complexes of Actinomycin D with pdGpdC. The deoxydinucleotide pdGpdC binds to actinomycin D to form a miniature double-helical complex which may be used as a model for the binding of the drug to DNA (Krugh, 1972; Krugh and Neely, 1973a,b; Krugh and Chen, 1975; Patel, 1974a; and references therein). The temperature dependence of the ^{31}P NMR spectrum of a 2:1 pdGpdC–actinomycin D solution is presented in Figure 3. The complexation shifts ($\Delta\delta$) for this system are listed in Table III. The significant features in this experiment are: (a) a large downfield shift of 1.4 ppm (26 °C) to 1.7 ppm (4 °C) for the internucleotide phosphate resonance of pdG*p*dC upon complexation with actinomycin D; (b) an increase in the line width of the internucleotide phosphate resonance from a line width of ~5 Hz (26 °C) to ~27 Hz (4 °C) as the temperature is lowered; and (c) an upfield shift of the terminal phosphate resonance (~0.2 ppm) upon binding, which is accompanied by line broadening at both 4 and 26 °C. These results are in general agreement with Patel's previous study (Patel, 1974a).

In order to establish the chemical shifts of the bound inter-

TABLE III: Complexation Shifts ($\Delta\delta$) of the Internucleotide Phosphate Resonances in the Deoxydinucleotide–Actinomycin D Solutions.

Nucleotide	Nucleotide/drug	T (°C)	$\Delta\delta^a$(ppm)
pdGpdC	2:1	26	−1.42
pdGpdC	2:1	4	−1.67
pdGpdC	2:1[b]	−18	−1.69; −2.43
pdCpdG	2:1	4	+0.13
pdGpdT	2:1	7	−0.17
pdGpdT	1:1	8	−0.20
pdApdC	1:1	8	−0.05
pdGpdT + pdApdC	1:1:1[c]	22	−0.22(GT); −0.18(AC)
pdGpdT + pdApdC	1:1:1[c]	8	−0.25(GT); −0.37(AC)
pdGpdT + pdApdC	1:1:1[b]	−16	−0.18(GT); −0.75(AC)
pdCpdA	1:1	22	+0.05
pdGpdT + pdCpdA	2:2:1	7	−0.17(GT); ~0(CA)
pdGpdT + pdCpdA	1:1:1	7	−0.27(GT); +0.03(CA)
pdGpdG	1:1	24	−0.08
pdCpdC	1:1	22	~0
pdGpdG + pdCpdC	1:1:1	24	−0.35(GG); −0.07(CC)
pdGpdG + pdCpdC	1:1:1	4	−0.13(CC)[d]
pdGpdA	2:1	7	−0.15
pdGpdA	1:1	7	−0.24
pdGpdA	0.5:1	7	−0.37

[a] Complexation shifts, $\Delta\delta$(ppm), are determined at the indicated temperatures using $\Delta\delta$ = δ(nucleotide solution) − δ(nucleotide–actinomycin D solution) under the conditions listed in the table. Negative values indicate downfield complexation shifts. [b] 30% (v/v) methanol-d_4/D_2O solvent, pH meter reading = 7.4. [c] Identical complexation shifts ($\Delta\delta$) were observed in both 99.9% D_2O and in 30% (v/v) methanol-d_4/D_2O solvents. [d] The internucleotide phosphate resonance from pdGpdG was broadened into the baseline.

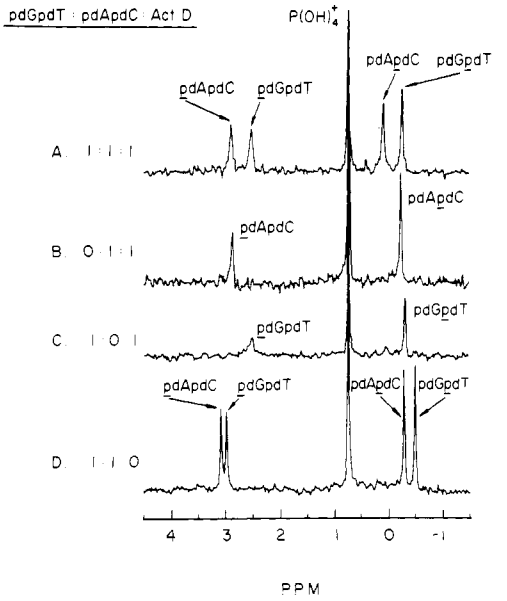

FIGURE 4: 40.48 MHz ^{31}P Fourier transform NMR spectra at 8 °C of (A) 1:1:1 pdGpdT–pdApdC–actinomycin D, (B) 1:1 pdApdC–actinomycin D, (C) 1:1 pdGpdT–actinomycin D, and (D) 1:1 pdGpdT–pdApdC solution mixtures. The actinomycin D concentration was 4.5 mM in D_2O, and the $P(OH)_4^+$ reference is 0.70 ppm downfield of 15% H_3PO_4.

nucleotide phosphate resonance of pdG*p*dC, a 2:1 pdGpdC–actinomycin D solution in a 30% (v/v) methanol-d_4/D_2O solvent was examined at −18 °C (Figure 3A). The addition of methanol to the 2:1 pdGpdC–actinomycin D solution at room temperature resulted in a ~0.7 ppm shift upfield of the terminal phosphate resonance, whereas the internucleotide phosphate resonance was shifted upfield ~0.13 ppm. The change in the chemical shift of the phosphate resonances is presumably either a solvent effect or a pH effect. To avoid possible overlap of the terminal and internucleotide phosphate peaks at low temperatures, the pH of the methanol-d_4/D_2O solvent was adjusted to a pH meter reading of 7.4.

The spectrum in Figure 3A at −18 °C clearly shows the presence of two internucleotide pdG*p*dC resonances, each with approximately a 5-Hz line width. The complexation shifts ($\Delta\delta$) for the formation of a 2:1 pdGpdC–actinomycin D complex are −1.69 and −2.43 ppm (Table III).

Circular dichroism spectra of the 2:1 pdGpdC–actinomycin D solution in the 30% (v/v) methanol-d_4/D_2O solvent were recorded at the same temperatures as in the ^{31}P NMR experiments. The characteristics of the circular dichroism spectra were essentially the same as those observed for a 0.134 mM actinomycin D +2.42 mM pdGpdC mixture in an H_2O solvent (Auer et al., unpublished data). At −18 °C, a large negative band at 383 nm ($\epsilon_l - \epsilon_r$ = −9.88) with smaller overlapping negative bands at ~450 ($\epsilon_l - \epsilon_r$ = −1.70) and 470 nm ($\epsilon_l - \epsilon_r$ = −2.35) are observed for the pdGpdC–actinomycin D complex. The locations of the extrema in this spectrum are essentially the same as those observed in the DNA–actinomycin D complex (Yamaoka and Ziffer, 1968; Homer, 1969).

A separate study of a 2:1 pdCpdG–actinomycin D solution at both 26 and 4 °C showed a small upfield complexation shift ($\Delta\delta$ = +0.13 ppm) of the resonance from the internucleotide phosphate group. The chemical shift of the terminal phosphate of pdCpdG did not change upon complex formation with actinomycin D. Neither complexation nor temperature variation brought about any significant line broadening of either resonance in this solution. The previous proton magnetic resonance data have clearly showed that pdCpdG forms a stacked (i.e., a nonintercalated) complex with actinomycin D (Krugh and Neely, 1973b; Krugh and Chen, 1975). The circular dichroism spectrum of this solution also indicates the formation of a stacked complex (Auer et al., in preparation).

The large deshielding of the internucleotide phosphate resonance in the 2:1 pdGpdC–actinomycin D complex results from the intercalation of the drug between the base pairs of the dinucleotides. The small upfield shift of the internucleotide phosphate of pdCpdG in the 2:1 pdCpdG–actinomycin D complex is associated with the formation of the stacked complex. The potential usefulness of ^{31}P NMR was investigated further by studying solutions of complementary and noncomplementary deoxydinucleotide mixtures with actinomycin D.

Actinomycin D + pdGpdT + pdApdC Complexes. The ^{31}P spectra of a 1:1 mixture of the *complementary* deoxydinucleotides pdGpdT–pdApdC in the absence and presence of actinomycin D are shown in Figure 4. Definitive assignments of all of the phosphorus resonances in the 1:1 pdGpdT–pdApdC mixture (Figure 4D) were made by a comparison of the free dinucleotide resonances in separate pdGpdT and pdApdC solutions (Table I). Final assignments of the chemical shifts were made by adding excess pdApdC to the 1:1 mixture and observing the increase in the intensity of these nucleotide

resonances. The chemical shifts of the internucleotide phosphate resonances are not significantly changed upon mixing of the two complementary dinucleotides. The addition of actinomycin D to a pdGpdT solution causes a downfield shift of the internucleotide phosphate resonance ($\Delta\delta = -0.20$ ppm), as seen in Figure 4C for a 1:1 pdGpdT–actinomycin D mixture. In contrast, the addition of actinomycin D to a pdApdC solution causes only a slight downfield shift ($\Delta\delta = -0.05$ ppm) of the internucleotide phosphate of pdApdC (Figure 4B). In both of these 1:1 mixtures of actinomycin D with either pdGpdT or pdApdC, we observe upfield complexation shifts for the terminal phosphate resonances (Figure 4B, C).

The ^{31}P NMR spectrum of the 1:1:1 pdGpdT–pdApdC–actinomycin D mixture is presented in Figure 4A, with the corresponding complexation shifts given in Table III. The internucleotide phosphate resonances in this mixture were assigned by the addition of excess pdGpdT. The complexation shifts of the internucleotide phosphate resonances at −18 °C are −0.18 and −0.75 ppm for pdG*p*dT and pdA*p*dC, respectively (Table III). In the presence of actinomycin D, the terminal phosphates of both of the deoxydinucleotides in the 1:1:1 mixture have approximately the same chemical shift changes upon binding as in the separate 1:1 dinucleotide–actinomycin D mixtures.

Actinomycin D + pdGpdT + pdCpdA Complexes. The ^{31}P complexation shifts for the *noncomplementary* deoxydinucleotide pdGpdT + pdCpdA solutions with actinomycin D are listed in Table III. Note that only the pdGpdT phosphorus resonances exhibit a change in their chemical shifts upon the addition of actinomycin D to an equimolar mixture of pdGpdT + pdCpdA. The magnitudes of these shifts for both the terminal and internucleotide phosphate resonances of pdGpdT are approximately the same as those observed in the pdGpdT + actinomycin D solutions. The chemical shift assignments of the internucleotide phosphate resonances in the 1:1:1 pdGpdT–pdCpdA–actinomycin D solution were verified by the addition of pdGpdT.

Actinomycin D + pdGpdG + pdCpdC Complexes. The complexation shifts of the internucleotide phosphate resonances of pdGpdG and pdCpdC that are observed upon the addition of actinomycin D are listed in Table III. Prior to the addition of actinomycin D, the pdGpdG resonances in the 1:1 pdGpdG–pdCpdC solution were much lower in intensity than the pdCpdC resonances. Thin-layer chromatographic analysis of these dinucleotides showed only a minor impurity in the pdGpdG solution. Ultraviolet absorption spectra verified the concentrations of the solutions and no anomalies were observed in the absorption spectra. The variations in the ^{31}P NMR intensities may be due to differences in relaxation rates or nuclear Overhauser effects on the phosphorus nuclei for these dinucleotides, possibly due to aggregation of the pdGpdG.

At 24 °C, a complexation shift of −0.08 ppm is observed for the internucleotide phosphate resonance of pdGpdG in a 1:1 pdGpdG–actinomycin D solution (Table III), while the pdCpdC resonances are unchanged in a 1:1 pdCpdC–actinomycin D solution. In an equimolar mixture of both dinucleotides with actinomycin D (i.e., a 1:1:1 mixture), the complexation shift of pdGpdG is −0.35 ppm. A small complexation shift of −0.07 ppm is observed for pdCpdC in this 1:1:1 mixture. Upon lowering the temperature of the actinomycin D–pdGpdG–pdCpdC mixture, the pdG*p*dG resonance is broadened into the the baseline while the internucleotide resonance of pdC*p*dC is broadened and shifted further downfield ($\Delta\delta =$ −0.13 ppm, Table II). The small downfield complexation shift for the internucleotide phosphate of pdC*p*dC ($\Delta\delta = -0.13$ ppm at 4 °C), which occurs only in the 1:1:1 pdGpdG–pdCpdC–actinomycin D solution, indicates that pdCpdC binds to actinomycin D only in the presence of pdGpdG. These observations are consistent with our previous data which showed that pdGpdG changes from a predominantly stacked complex in the 1:1 pdGpdG–actinomycin D solution to an intercalated complex in the 1:1:1 pdGpdG–pdCpdC–actinomycin D solution (Krugh et al., 1977; and Chiao, 1976).

Actinomycin D Complex with pdGpdA. The phosphorus-31 spectra of pdGpdA as well as 0.5:1, 1:1, and 2:1 pdGpdA–actinomycin D mixtures were recorded. The largest complexation shift was observed in the 0.5:1 solution ($\Delta\delta = -0.37$ ppm), as compared to a value of $\Delta\delta = -0.15$ ppm in the 2:1 solution. It is instructive to compare these results with the proton magnetic resonance data (Figure 7 of Krugh and Neely, 1973b), where the unusual titration data for the 4-CH_3 resonance indicated that the conformation of the pdGpdA in the pdGpdA–actinomycin D complex changed during the titration.

Discussion

The most striking feature in the ^{31}P NMR spectra of all of the drug–dinucleotide complexes reported to date is the large downfield complexation shifts of the internucleotide phosphates when actinomycin D intercalates at a (dGpdC)·(dGpdC) sequence. Patel has previously reported these large shifts in the actinomycin D complexes with pdGpdC and other oligonucleotides (Patel, 1974a,b; 1976). All of these actinomycin D complexes presumably involve intercalation of the phenoxazone ring of actinomycin D between adjacent G·C base pairs (i.e., intercalation at a (dGpdC)·(dGpdC) sequence) to form miniature double-helical complexes. If these large downfield complexation shifts are generally characteristic of the formation of an intercalated complex (as suggested by Patel), then we might expect to observe similar complexation shifts for the ethidium bromide and 9-aminoacridine complexes with the dinucleotides, since both of these drugs intercalate into DNA (e.g., see Lerman, 1961, 1963; Luzzati et al., 1961; Cohen and Eisenberg, 1969; Bauer and Vinograd, 1968, 1970; Waring, 1970). Evidence from previous visible, fluorescence, circular dichroism, proton magnetic resonance, and kinetic studies of 2:1 CpG–ethidium bromide or 2:1 pdCpdG–ethidium bromide solutions (Krugh et al., 1975; Krugh and Reinhardt, 1975; Krugh et al., in preparation; Davanloo and Crothers, 1976) show that ethidium acts as a nucleation center and binds two CpG or pdCpdG molecules through a cooperative stepwise association to form a miniature intercalated complex. Since we observe only relatively small (~0.2 ppm) ^{31}P complexation shifts for these complexes (Table II), we conclude that the formation of an intercalated complex is not always accompanied by a large deshielding of the ^{31}P resonances from the phosphate groups at the intercalation site. However, the complexation shifts observed in the ethidium bromide and 9-aminoacridine complexes do provide a monitor of complex formation.

Actinomycin D Complexes. In the mixed solvent system ($CD_3OD + D_2O$) at −18 °C, the chemical exchange of the 2:1 pdGpdC–actinomycin D complex is sufficiently slow ($\tau > 100$ ms) so that individual resonances are observed for the two phosphate groups at the intercalation site (Figure 3A, and Table III). The complexation shifts of these two phosphate resonances are quite similar to two of the phosphate resonances in the 2:1 nucleotide–actinomycin D complexes in which the nucleotides were d(CpGpCpG), d(CpGpCpGpCpG), and

d(ApTpGpCpApT) observed by Patel (1974b, 1976). The main difference in the ^{31}P spectra of the actinomycin D complex with pdGpdC when compared to the tetra- and hexanucleotide complexes is that the dinucleotide–actinomycin D complexes are thermodynamically less stable (as expected), which necessitated the use of the mixed solvent system to allow the spectra to be recorded in the slow-exchange region.[1]

The question arises as to whether these complexation shifts are unique for actinomycin D binding to a (dGpdC)·(dGpdC) sequence, or whether it is a general property associated with the intercalation of actinomycin D into double-stranded nucleic acids. The internucleotide phosphate resonance of pdGpdT in the 1:1:1 pdGpdT–pdApdC–actinomycin D mixture at −16 °C is shifted downfield −0.18 ppm, while the complexation shift of the internucleotide phosphate resonance of pdApdC in this 1:1:1 solution of complementary dinucleotides is −0.75 ppm. The different complexation shifts for these resonances also suggest an asymmetric environment at the intercalation site. The substituents on the phenoxazone ring of actinomycin D result in an asymmetry of the ring, but it should also be noted that the phenoxazone ring is slightly *nonplanar* and thus it would not be surprising if the geometries of the phosphodiester linkages at the intercalation site are not identical. One possible explanation for the different complexation shifts that are observed in the pdGpdC–actinomycin D spectra and the pdGpdT–pdApdC–actinomycin D spectra is that when actinomycin D intercalates into the (dGpdC)·(dGpdC) sequence *both* of the guanine 2-amino groups form a hydrogen bond with the threonine carbonyl (e.g., see Sobell and Jain, 1972). On the other hand, when actinomycin D intercalates into a (dGpdT)·(dApdC) sequence there is only *one* guanine 2-amino group to form a hydrogen bond with a threonine carbonyl group of actinomycin D. The spatial restrictions that arise from the formation of two hydrogen bonds may possibly result in geometries of the phosphate groups that depend slightly upon the nucleotide sequence at the intercalation site. Although this last interpretation is somewhat speculative at the present time, it does provide a basis for suggesting future experiments with deoxytetranucleotides and deoxyhexanucleotides which contain only a single guanine base at the intercalation site, since these complexes will hopefully provide further insight toward a more quantitative understanding of the actinomycin D–DNA complex as well as the complexation shifts observed in ^{31}P NMR spectra.

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[1] Note Added in Proof. After this manuscript was submitted a paper appeared in which D. J. Patel ((1976), *Biochim. Biophys. Acta 442,* 98) reexamined the ^{31}P NMR spectra of the 2:1 pdG-dC–actinomycin D complex at 145.7 MHz. With this "supercon" spectrometer the individual resonances of the internucleotide phosphates were resolvable in aqueous solution at 1 °C.