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Proton and Phosphorus Nuclear Magnetic Resonance Studies of an Oligothymidylate Covalently Linked to an Acridine Derivative and of Its Binding to Complementary Sequences

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Received May 21, 1984; Revised Manuscript Received August 23, 1984

ABSTRACT: An oligodeoxynucleotide containing four thymines and covalently attached to an acridine derivative through its 3′-phosphate [$(Tp)_4(CH_2)_5Acr$] was synthesized. Its conformation in solution was investigated by proton magnetic resonance. Both intramolecular interactions between the acridine dye and thymines and intermolecular interactions were demonstrated. Both proton and phosphorus magnetic resonances were used to study the specific interaction of $(Tp)_4(CH_2)_5Acr$ with poly(rA) and $(Ap)_3A$. The results were compared to those obtained when the acridine-containing substituent was replaced by an ethyl group attached to the 3′-phosphate of the oligothymidylate. The acridine dye strongly stabilized the complexes formed with both poly(rA) and $(Ap)_3A$. Upfield shifts of both adenine and acridine proton resonances were observed in the complexes. These results were ascribed to an intercalation of the acridine ring between A·T base pairs of the duplex structure formed by the oligothymidylate with its complementary oligoadenylate sequence. An analysis of proton and phosphorus chemical shifts as well as measurements of T_1 relaxation times at different temperatures allowed us to propose several structures for the complexes formed by $(Tp)_4(CH_2)_5Acr$ with its complementary sequence.

The control of gene expression in both procaryotes and eucaryotes requires molecules that bind selectively to specific nucleic acid sequences during either transcription of DNA or translation of messenger RNAs. These processes are usually regulated by specific nucleic acid binding proteins. The interactions between functional groups in protein–nucleic acid complexes have been recently reviewed (Hélène & Lancelot, 1982). In some cases, however, regulation can be achieved by a nucleic acid fragment that is complementary (at least in part) to the control sequence (Mizuno et al., 1984).

0006-2960/85/0424-2521$01.50/0

Binding of an oligonucleotide to its complementary sequence is a highly specific process that rests upon hydrogen-bond formation between complementary bases. In order to achieve a higher affinity without loosing the specificity of base-pair formation, oligonucleotides can be modified in such a way as to introduce additional interactions with the target sequence. We have recently described the synthesis of a new family of molecules in which a DNA intercalating agent is covalently linked to an oligodeoxynucleotide (Asseline et al., 1983, 1984a,b). A derivative of 9-aminoacridine (2-methoxy-6-chloro-9-aminoacridine) was covalently attached to the 3′- or 5′-phosphate of an oligothymidylate via a polymethylene linker. Absorption and fluorescence spectroscopies demonstrated that these molecules interact in a specific way with their complementary sequence [poly(rA), poly(dA), oligo(rA), and oligo(dA)] and that the presence of the acridine dye strongly stabilizes the complexes when compared with the same oligothymidylate lacking the acridine substituent. These substances are therefore sequence-specific molecules with high affinity for their target sequence. They could be used to regulate gene expression provided the target sequence is available for hydrogen-bond formation with the bases of the oligonucleotide. This is obviously so if the sequence is located in a single-stranded nucleic acid but also if it is part of a transiently opened region of double-stranded DNA during replication or transcription processes.

In order to obtain more information on the structure of the complexes formed by an oligothymidylate bearing an acridine derivative and on the interactions engaged by the acridine ring with nucleic acid bases, we undertook a nuclear magnetic resonance investigation of $(Tp)_4(CH_2)_5Acr$, a tetrathymidylate linked to the 9-amino group of 2-methoxy-6-chloro-9-aminoacridine via a pentamethylene linker. Here we report proton and phosphorus NMR results of its interactions with poly(rA) and $(Ap)_3A$ in aqueous solutions at pH 7. These results show that the acridine ring engages stacking interactions with A·T base pairs in the duplex structure formed by the oligothymidylate with its complementary sequence.

Experimental Procedures

The syntheses of the oligonucleotides used in this study have been described elsewhere (Asseline et al., 1983). Covalent attachment of 2-methoxy-6-chloro-9-aminoacridine to the oligodeoxynucleotide involved a chain of five methylene groups linking the 3′-phosphate of the tetranucleotide to the 9-amino group of acridine. This compound will be abbreviated as $(Tp)_4(CH_2)_5Acr$. Oligothymidylates substituted by an ethyl group on the 3′-phosphate [$(Tp)_4Et$ and $(Tp)_8Et$] were also prepared to compare their binding to complementary sequences with that of $(Tp)_4(CH_2)_5Acr$.

The tetraribonucleotide $(Ap)_3A$ and poly(rA) were obtained from P-L Biochemicals and used without further purification. The oligonucleotides $(Tp)_4(CH_2)_5Acr$, $(Tp)_4Et$, and $(Tp)_8Et$ were purified by high-pressure liquid chromatography (HPLC). All the investigated compounds were passed through a Chelex-100 column at pH 7.0 to remove paramagnetic impurities, then lyophilized, and dissolved in a pH 7.0 buffer (^2H_2O) containing 0.01 M sodium phosphate, 0.1 M NaCl, and 1 mM ethylenediaminetetraacetic acid (EDTA).

Proton NMR spectra were recorded at three different frequencies on a Bruker WH 90, a Bruker WA 300, or a Bruker WM 400 Fourier-transform spectrometer. ^{31}P NMR spectra were recorded on a Bruker WH 90 and a Bruker WA 300 operating at 36.4 and 121.5 MHz, respectively. The samples were placed in a 5-mm NMR tube, and the spectra were calibrated with respect to an internal reference of 4,4-dimethyl-4-silapentane-1-sulfonate (DSS) for proton spectra and of trimethyl phosphate (TMP) for phosphorus spectra. Proton relaxation times (T_1) were obtained by the inversion recovery method (180°–τ–90°) at 90 and 300 MHz on degassed solutions contained in sealed tubes. The T_1 relaxation times were computed by using a least-squares program to fit the three parameters I_0, α, and T_1 in the equation $I(t) = I_0[1 + \alpha \exp(-t/T_1)]$. Absorption spectra were recorded with a Cary 218 spectrophotometer. All concentrations will be expressed as moles of nucleotide per liter.

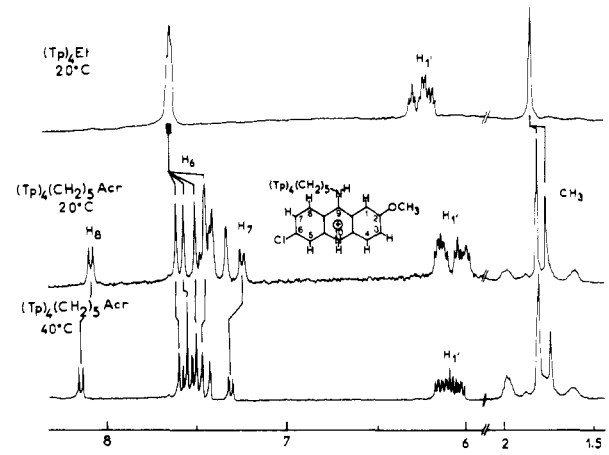

FIGURE 1: The 400-MHz ^1H NMR spectra of 2 mM $(Tp)_4(CH_2)_5Acr$ at 40 (lower spectrum) and 20 °C (middle) and of 2 mM $(Tp)_4Et$ at 20 °C (upper spectrum).

Results

Conformation of $(Tp)_4(CH_2)_5Acr$. The 400-MHz proton NMR spectrum of $(Tp)_4(CH_2)_5Acr$ is shown in Figure 1. The assignment of the proton resonances of the acridine ring was made as already published (Barbet et al., 1976).. The ^1H NMR spectrum of $(Tp)_4(CH_2)_5Acr$ was compared to that of the parent compound $(Tp)_4Et$ where the 3′-phosphate has been substituted by an ethyl group (Figure 1). The four H_6 protons of $(Tp)_4Et$ as well as the four methyl groups are nearly equivalent. In contrast, the four H_6 resonances are clearly separated in the $(Tp)_4(CH_2)_5Acr$ spectrum. Three methyl groups are nearly equivalent; the fourth one is shifted upfield. These results indicate that the four thymines in $(Tp)_4(CH_2)_5Acr$ experience different magnetic environments as a result of the attachment of the acridine ring to the 3′-phosphate. The methyl group that resonates at higher field can be ascribed to the base that is closer to the acridine ring (thymine-4).

Substitution of the 3′-phosphate group by the aliphatic chain bearing the acridine dye induces an upfield shift (0.15–0.25 ppm) of the $H_{1'}$ deoxyribose resonances. The sum of the sugar coupling constants $J_{1'2'} + J_{1'2''}$ is expected to be 7.5 Hz for a $C_{3'}$ endo conformation (^3E) and 15.6 Hz for a $C_{2'}$ endo conformation (^2E) (Karplus, 1969; Sarma, 1980). The observed values are 13.5 and 14.0 Hz for $(Tp)_4Et$ and $(Tp)_4(CH_2)_5Acr$, respectively. These results indicate a major contribution of an ^2E conformation of the deoxyribose in both $(Tp)_4Et$ and $(Tp)_4(CH_2)_5Acr$.

Lowering the temperature leads to an upfield shift of the H_8 and H_7 protons of the acridine ring as well as several shifts and changes in the spectra of other dye protons (Figure 1). The resonance lines of the H_6 protons and of the methyl groups of thymines are much less affected. Since the ring current effect of thymine is small (Giessner-Prettre et al., 1976) as compared with that of 2-methoxy-6-chloro-9-aminoacridine

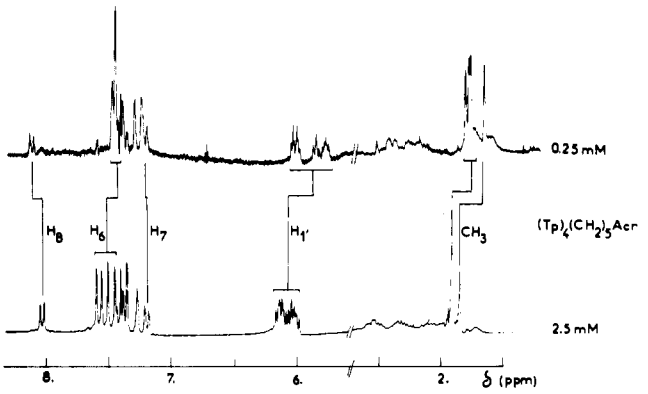

FIGURE 2: The 300-MHz ^1H NMR spectra of $(Tp)_4(CH_2)_5Acr$ at two concentrations (0.25 and 2.5 mM) at 21 °C.

(Barbet et al., 1976), the upfield shift of the dye aromatic protons suggests self-association of the acridine rings, i.e., intermolecular interactions between dye-substituted oligonucleotides, rather than an intramolecular conformational change. Lowering the information of $(Tp)_4(CH_2)_5Acr$ from 2.5 to 0.25 mM (Figure 2) leads to small downfield shifts of the proton resonances of the acridine ring together with upfield shifts of the thymine protons. This result indicates that dye–dye interactions are decreased when the concentration is lowered and that intramolecular interactions between the aromatic rings of acridine and thymines are enhanced.

Binding of $(Tp)_4(CH_2)_5Acr$ to Poly(rA). The binding of $(Tp)_4(CH_2)_5Acr$ to poly(rA) was followed by both absorption and ^1H NMR spectroscopy. As already reported (Asseline et al., 1983, 1984b), the visible absorption spectrum of $(Tp)_4(CH_2)_5Acr$ was markedly modified upon interaction with poly(rA). A strong hypochromism of the main acridine absorption band was observed together with the appearance of a new absorption at long wavelengths (≃470 nm). Plotting absorbance at 425 nm vs. the poly(rA) to acridine ratio gave a stoichiometry of 4, indicating that four A·T base pairs are formed in the complex (Asseline et al., 1984). The melting temperature was determined at the concentrations used in the ^1H NMR experiments (8.0 mM nucleotide). A value of 51 °C was obtained.

Increasing amounts of $(Tp)_4(CH_2)_5Acr$ were added to a 8.0 mM poly(rA) solution, and the ^1H NMR spectra of each mixture was recorded between 70 and 5 °C. At high temperature (70 °C) the ^1H NMR spectrum of the mixtures was the superposition of the spectra of the two components. Upon lowering the temperature from 70 to 42 °C, the H_2 resonance line of adenine was shifted upfield as compared with that of free poly(rA) while the line width was increased (Figure 3). For thymine to adenine ratios lower than 0.5, the H_2 resonance was then shifted downfield when the temperature was decreased below 42 °C, until it coincided with that observed for poly(rA) alone. The H_2 resonance line width decreased in the same temperature range and became very similar to that of poly(rA) below 20 °C. When the ratio of thymine to adenine concentrations was higher than 0.5, the line width increased so much at temperatures below 40 °C (>60 Hz at 90 MHz) that it became nearly impossible to locate the H_2 resonance.

A similar behavior was observed for the H_8 and $H_{1'}$ resonances even though the changes in chemical shifts and line widths were much smaller than for H_2 (see Figure 3). The areas under the resonance lines of H_8, H_2, and $H_{1'}$ were determined as a function of temperature for poly(rA) and poly(rA)–$(Tp)_4(CH_2)_5Acr$ mixtures. The area under the DSS peak was used as an internal reference. From 70 to 42 °C the area under the resonance lines of the mixtures was indistinguishable from that of poly(rA) alone. This did not hold below 40 °C where the relative area in the mixtures decreased. When the thymine to adenine concentration ratio was 0.5, the area under the H_8, H_2, and $H_{1'}$ lines at 10 °C was about 50% of that of poly(rA) alone. For all mixtures there was a good agreement between the loss of integrated area and the thymine to adenine ratio.

For poly(rA) alone the H_2 line width increased from 0.1 to ≃10 Hz when the temperature decreased from 70 to 10 °C, but the integrated intensity remained constant. This result indicates that a rapid exchange takes place between the different conformations of poly(rA) with the broadening resulting from a decreased mobility of the bases and of the polymer backbone. In the temperature range 42–70 °C, addition of $(Tp)_4(CH_2)_5Acr$ leads to complex formation with a rapid exchange between bound and free regions of poly(rA). Below

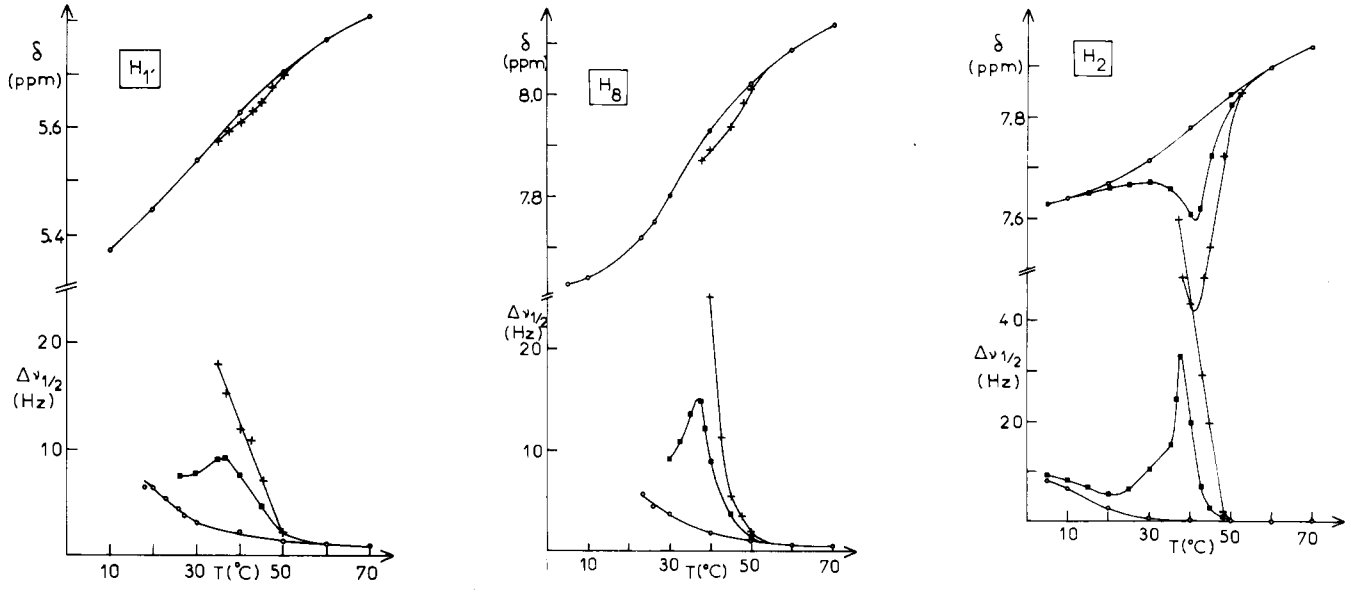

FIGURE 3: Temperature dependence of chemical shifts (δ) and line width ($\Delta\nu_{1/2}$) for the H_2, H_8, and $H_{1'}$ protons of 8.0 mM poly(rA) alone (○) and in the presence of $(Tp)_4(CH_2)_5Acr$ at 2.3 (■) and 4.7 mM (+). The melting temperature measured by absorption spectrophotometry was 46 °C for the solution containing 4.7 mM $(Tp)_4(CH_2)_5Acr$. The value obtained for the 1:1 complex [8 mM $(Tp)_4(CH_2)_5Acr$] was 51 °C (see text).

Table I: Comparison of Experimental Upfield Shifts ($\Delta\eta_{exp}$) Observed upon Complex Formation between Poly(rA) and $(Tp)_4(CH_2)_5Acr$ or $(Tp)_8Et$ with Those Computed for A, B, and D Double-Helical Conformations

		$\Delta\delta$ (isolated species)[b]	$\Delta\delta_{exp}$ (complexes)[c]		$\Delta\delta_{The}$[d]		
	δ_m[a]	poly(rA) or $(Tp)_4(CH_2)_5Ac$	poly(rA) + $(Tp)_8Et$	poly(rA) + $(Tp)_4(CH_2)_5Ac$	A form	B form	D form
$H_2(A)$	8.28	0.66	1.03	1.3 ± 0.1	1.0	1.15	1.5
$H_8(A)$	8.36	0.74	0.76	0.56 ± 0.05	0.5	0.2	0.1
$H_{1'}(A)$	6.11	0.76	0.55	0.55 ± 0.05	0.08	0.23	
$H_6(T)$	7.68	0.23–0.13–0.11, 0.07	0.17	*e*	0.05	0.0	0.0
$CH_3(T)$	1.92	0.12–0.18	0.33	0.42	0.1	0.05	0.05
$H_{1'}(T)$	6.27	0.05	0.12	0.15	0.15	0.07	

[a] δ_m = chemical shifts observed in adenine 5′-monophosphate (AMP) or thymidine 5-monophosphate (TMP) at 70 °C. [b] Difference in chemical shifts (upfield shifts) between poly(rA) or $(Tp)_4(CH_2)_5Acr$ at 5 °C and AMP or TMP. [c] Difference in chemical shifts between fully bound and free species calculated from eq 1. [d] $\Delta\delta_{The}$ = upfield shift computed from the data of Patel & Tonelli (1975) for stacked A.T base pairs in different conformations as compared to isolated nucleotides (AMP and TMP). The values computed for $H_{1'}$ were obtained according to the data of Arter & Schmidt (1976). [e] The broadening of the resonances lines upon complex formation as well as the overlap with the protons of acridine did not allow us to assign the H_6 resonances in the complex.

Table II: Variation in Chemical Shifts between 10 and 60 °C for Proton Resonances of Adenines and Thymines in $(Tp)_4(CH_2)_5Acr$, $(Ap)_3A$, and Their Equimolecular Mixture (2.3 mM:2.3 mM) in D_2O (pH 7.0), 0.25 M NaCl, and 10 mM Sodium Phosphate[a]

	$H_8(A)$				$H_2(B)$				$H_6(T)$	$CH_3(T)$		Ac			
	1	2	3	4	1	2	3	4	1–4	1–3	4	OCH_3	1, 3, 4, 5	7	8
$\Delta\delta_f$	−0.03	0.23	0.28	0.18	0.27	0.23	0.19	0.08	0.03, 0.00, 0.05, 0.10	0.00	0.02	0.00	0.05–0.10	0.09	0.11
$\Delta\delta_b$	0.15	0.27	0.34	0.31	0.58	0.64	0.67	0.48	0.00–0.10	0.00–0.10	0.08	0.41	0.67–0.80	0.55	0.67

[a] $\Delta\delta_f$ and $\Delta\delta_b$ refer to the free and bound state, respectively. Upfield shifts are positive. Bases in oligonucleotides are numbered from the 5′- to the 3′-end.

40 °C all available results [decrease in integrated intensity, downfield shift, and sharpening of the poly(rA) resonance lines in the complex] indicate that the rate of exchange between free and bound regions of poly(rA) reaches values that are of the same order of magnitude as $\pi(|\nu_b - \nu_f|)$, where ν_b and ν_f are the resonance frequencies of the investigated proton (H_2, H_8, or $H_{1'}$) in the bound and the free state, respectively. When the exchange rate becomes much smaller than $\pi(|\nu_b - \nu_f|)$, two resonances should be observed for each proton: one due to free regions of poly(rA) and one to the covered regions, with respective contributions corresponding to the populations of the two species. At 10 °C, the H_2, H_8 and $H_{1'}$ resonances of the poly(rA)–$(Tp)_4(CH_2)_5Acr$ mixtures have the same chemical shifts as those of free poly(rA) (Figure 3), but their integrated intensity is decreased by an amount corresponding to that expected if each oligonucleotide covers four poly(rA) nucleotides. Only free regions contribute to the resonance intensity. The covered regions of poly(rA) are no longer observed because the line width is too large.

Several experiments were carried out at 400 MHz. The broadening of the resonance lines occurred at higher temperature than at 90 MHz. It was difficult to locate resonance lines below 60 °C. This result is in agreement with the interpretation given above for 90-MHz spectra. Due to the increase in $|\nu_b - \nu_f|$ at 400 MHz (as compared with 90 MHz), the region of intermediate exchange is shifted to higher temperatures when the rate of exchange between free and covered regions of poly(rA) becomes of the order of $\pi|\nu_b - \nu_f|$.

At 70 °C the ^1H NMR spectrum of a mixture of poly(rA) and $(Tp)_4(CH_2)_5Acr$ was the superposition of the individual spectra of the two components. When the temperature was lowered, the resonance lines of thymine (H_6 and CH_3) and of acridine (H_7, H_8, OCH_3) were shifted upfield and broadened. The behavior of the H_1, H_3, H_4, andd H_5 lines of acridine was difficult to investigate due to the overlap of many resonance lines in the downfield region. The H_6 resonance lines of thymines were identified by comparing the spectra obtained with and without irradiation of the methyl resonances. For thymine to adenine ratios smaller or equal to 1, all resonance lines were too broad to be observed at temperatures below $\simeq$50 °C.

Binding of $(Tp)_8Et$ to Poly(rA). The binding of $(Tp)_4(CH_2)_5Acr$ to poly(rA) is accompanied by upfield shifts and broadening of both adenine and thymine resonance lines as described above (see Table I). In order to determine the respective contributions of base pairing and acridine interaction, we investigated the binding of poly(rA) to $(Tp)_8Et$, where an ethyl group substitutes the 3′-phosphate group. This oligonucleotide was chosen because the melting temperature of its complex with poly(rA) is close to that of $(Tp)_4(CH_2)_5Acr$ than that of $(Tp)_4Et$ (see below). The ethyl substituent replaces the pentamethylene chain that links the acridine ring to the 3′-phosphate.

The binding of $(Tp)_8Et$ to poly(rA) is accompanied by upfield shifts and a broadening of the resonance lines of adenine H_2, H_8 and $H_{1'}$ and of thymine H_6, $H_{1'}$, and CH_3 protons (Table I). For a thymine to adenine ratio of 1, the line width of all these resonances reaches ca. 30 Hz at 20 °C whereas H_2, H_8, and $H_{1'}$, resonances of poly(rA) at the same temperature have line widths of 2.5, 5.0, and 6 Hz, respectively. The free oligonucleotide resonance lines have a width smaller than 1 Hz under the same conditions.

The binding of $(Tp)_8Et$ to poly(rA) is accompanied by an hypochromism at 260 nm. By use of Job's (1928) procedure, a 1:1 stoichiometry (T:A) was determined for this complex. As expected, the dissociation was a cooperative process. The melting temperature (T_m) determined at the oligonucleotide concentration used in the ^1H NMR experiments (10 mM nucleotide/L) was 32 °C in agreement with the ^1H NMR results (Figure 4). The T_m value for $(Tp)_4(CH_2)_5Acr$ was 51 °C at 8 mM (see above).

Binding of $(Tp)_4(CH_2)_5Acr$ to $(Ap)_3A$. Addition of $(Ap)_3A$ to $(Tp)_4(CH_2)_5Acr$ at low temperature led to a general broadening of all the resonance lines and to an upfield shift of several resonances (Table II). The temperature dependence of the proton resonances in the mixture is shown in Figure 5. The assignments of the H_2 and H_8 protons of adenine in $(Ap)_3A$ alone or in the mixture at 60 °C (when the complex

Table III: T_1 Relaxation Times Measured at 300 and 90 MHz for Proton Resonances of Adenines and Thymines in the Mixture $(Tp)_4(CH_2)_5Acr$–$(Ap)_3A$ (2.3 mM:2.3 mM) at 21 and 45 °C[a]

		$(Ap)_3A$			$(Tp)_4(CH_2)_5Acr$			Acr
		H_8	H_2	$H_{1'}$	H_6	CH_3	$H_{1'}$	7
21 °C	T_1 (s), 300 MHz	2.3	[b]	1.2	1.6	0.9	2.7	[c]
	T_1 (s), 90 MHz	0.39	1.6	0.26	[c]	0.55	0.48	[c]
	τ	1.2		0.9		0.25[d]	1.1	
	$\langle r \rangle$	0.74		0.69		[d]	0.77	
45 °C	T_1 (s), 300 MHz	1.3	3.7	0.8	0.94	0.82	0.76	1.6
	T_1 (s), 90 MHz	0.45	2.7	0.4	0.48	0.50	0.37	0.87
	τ	0.6	0.20	0.33	0.25	0.2	0.3	0.3
	$\langle r \rangle$	0.73	[e]	0.68	0.75	[d]	0.68	0.77

[a] The correlation time τ and the average of the distance between interacting spins $\langle r \rangle$ were computed by using eq 3 assuming that only one proton is participating in the dipole–dipole process of relaxation. The lifetimes have been measured for each H_8, H_2, and CH_3 proton. The best fit obtained by using a least-squares program showed that, within experimental error, the T_1 values are the same for the four bases. [b] T_1 was impossible to measure as a result of the broadening of the resonance lines due to chemical exchange. [c] T_1 was not determined due to the overlap with other resonance lines. [d] The relaxation processes of the methyl group are governed by the rotation of CH_3 around the C_5–CH_3 bond and the overall rotation off the molecule. [e] The computation of $\langle r \rangle$ has no meaning since quadrupolar relaxation by nitrogen atoms N_1 and N_3 is participating in the relaxation processes of the H_2 proton of adenine.

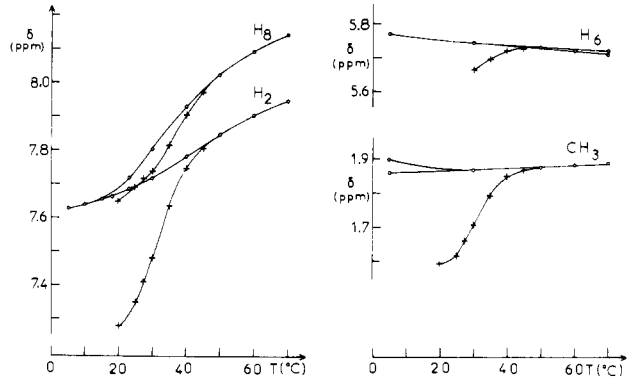

FIGURE 4: Temperature dependence of the chemical shifts of the H_2 and H_8 protons of 20 mM poly(rA) (left) and of the H_6 and CH_3 protons of 10 mM $(Tp)_8Et$ (right). Circles refer to isolated molecules and crosses to their 1:1 mixture.

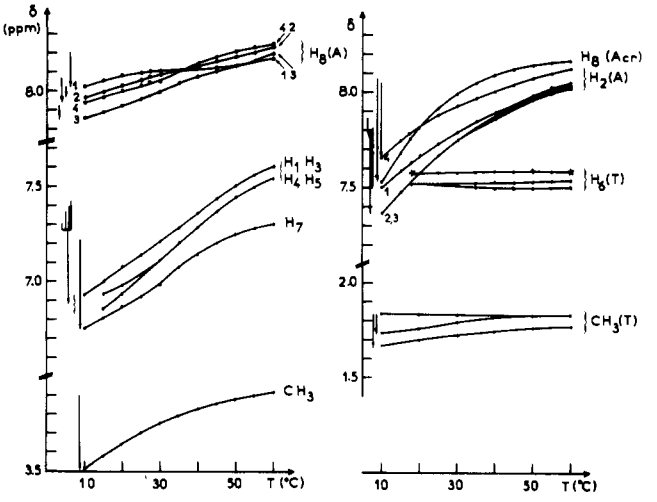

FIGURE 5: Temperature dependence of proton chemical shifts in the mixture $(Tp)_4(CH_2)_5Acr$–$(Ap)_3A$ (2.3 mM:2.3 mM). The arrows show the difference in chemical shifts between the uncomplexed and the complexed molecules at 10 °C.

is dissociated) were made as already published (Kroon et al., 1974). The coupled H_7 and H_8 protons (J = 9.4 Hz) of acridine have resonances well separated from other resonances and were easily assigned. The broadening of the resonance lines as well as the overlap with the H_6 protons of the thymines did not allow us to assign separately the H_1, H_3, H_4, and H_5 resonances of the acridine derivative. Even though complex formation was not complete at 10 °C, the binding of $(Ap)_3A$ to $(Tp)_4(CH_2)_5Acr$ led to an important upfield shift of all the aromatic resonances of adenine and acridine (Figure 5). The thymine methyl groups gave rise to three resonances in the 1:1 mixture at 10 °C (Figure 5). The resonance at 1.83 ppm accounted for two methyl groups and was not shifted as compared with free $(Tp)_4(CH_2)_5Acr$. The other two methyl resonances were shifted by 0.08 and 0.1 ppm. The four H_6 resonances were shifted by less than 0.02 ppm in the 1:1 mixture at 10 °C.

There are four sugar residues in each oligonucleotide. In the 1:1 mixture at 18 °C, the triplets corresponding to the $H_{1'}$ resonances of $(Tp)_4(CH_2)_5Acr$ were observed at 6.15, 6.13, 6.12, and 6.03 ppm whereas the doublets of the $H_{1'}$ resonances of the oligonucleotide $(Ap)_3A$ were observed at 5.79, 5.73, 5.64, and 5.63 ppm. The sum of the coupling constants $J_{1'2'} + J_{1'2''}$ was 11–12 Hz for the triplets whereas $J_{1'2'}$ was 3–4 Hz for the doublets. The sum $(J_{1'2'} + J_{1'2''})$ increased with temperature until a value of 14 Hz was reached at 60 °C when the complex was dissociated. As complex formation is not completed at 18 °C, the value of $J_{1'2'} + J_{1'2''}$ in the complex $(Tp)_4(CH_2)_5Acr$–$(Ap)_3A$ is certainly less than 11 Hz. This indicates that the predominant deoxyribose conformation shifts from $C_{2'}$ endo in the free oligodeoxynucleotide to partially $C_{3'}$ endo in the complex with $(Ap)_3A$.

Spin–lattice relaxation times (T_1) were measured at two frequencies (90 and 300 MHz) and at two different temperatures: 45 (where the complex is dissociated) and 21 °C (where partial association has taken place) (Table III). From these T_1 values the average distance $\langle r \rangle$ between the investigated proton and other relaxing protons as well as rotational correlation times has been calculated (see discussion).

In order to obtain more information on the structure of the complexes, attempts were made to measure nuclear Overhauser effects (NOE) and to record the resonances of exchangeable imino protons of A·T base pairs in H_2O solution. At low temperature (10 °C) line broadening due to self-association of the complexes prevented us from obtaining reliable data from both NOE measurements, and NH resonances could not be detected. At higher temperatures (>15 °C) when line broadening was no longer the limiting parameter, part of the complex was dissociated and in fast exchange with the separated molecules. Neither interresidue NOEs nor NH resonances could be detected at these temperatures.

Phosphorus Resonance of Backbone Phosphates. The proton noise decoupled 36.4-MHz ^{31}P magnetic resonance spectrum of $(Tp)_4(CH_2)_5Acr$ shows four well-separated resonances lines at 3.36, 3.95, 4.05, and 4.10 ppm at 50 °C (Figure 6). Three phosphates are covalently linked to two

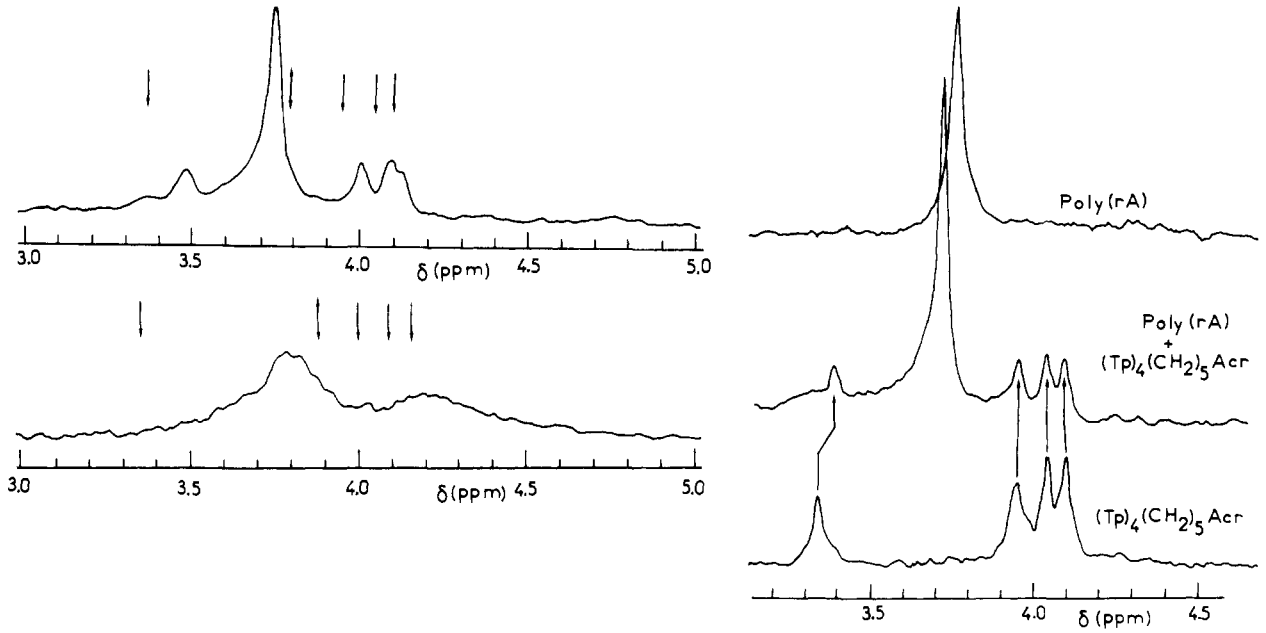

FIGURE 6: Proton noise decoupled ^{31}P spectra at 36.4 MHz. (Left) 18 mM poly(rA) (top), poly(rA)–$(Tp)_4(CH_2)_5$Acr (8 mM:4.7 mM) (middle), and 5 mM $(Tp)_4(CH_2)_5$Acr (bottom) at 50 °C. (Right) Mixture of poly(rA) and $(Tp)_4(CH_2)_5$Acr at 47 and 40 °C. The arrows indicate the position of the ^{31}P resonances of the isolated molecules at the same temperature [single arrow for $(Tp)_4(CH_2)_5$Acr and double arrow for poly(rA)].

deoxyriboses in positions 3′ and 5′ and have similar environments. They probably account for the last three resonances. Only one phosphate is covalently linked to an aliphatic chain; this last group was tentatively assigned to the resonance at 3.36 ppm. Addition of poly(rA) to $(Tp)_4(CH_2)_5$Acr led to an upfield shift of the resonance lines of the oligonucleotide phosphates (especially that linked to the acridine substituent) and a downfield shift of poly(rA) phosphates (Figure 6). These shifts depended on temperature and increased in magnitude from 70 to 40 °C. Below 40 °C, the broadening of the phosphate resonance lines was such that it became impossible to determine their respective positions.

In order to obtain supplementary information on this system, we have investigated a 1:1 mixture of $(Tp)_4(CH_2)_5$Acr and $(Ap)_3A$. The temperature dependence of the phosphorus chemical shifts in $(Tp)_4(CH_2)_5$Acr, $(Ap)_3A$, and their equimolar mixture is shown in Figure 7. At 10 °C, the phosphorus resonances observed at 4.35 and 4.27 ppm in free $(Tp)_4(CH_2)_5$Acr were downfield shifted by 0.19 and 0.10 ppm, respectively, in the mixture with $(Ap)_3A$. The other two phosphorus resonances observed at 3.42 and 4.18 ppm in the free oligonucleotide were shifted *upfield* by 0.24 and 0.02 ppm in the complex, respectively. Under the same experimental conditions, two phosphorus resonances of $(Ap)_3A$ were *downfield* shifted by 0.21 and 0.13 ppm while the third phosphorus resonance was *upfield* shifted by 0.14 ppm.

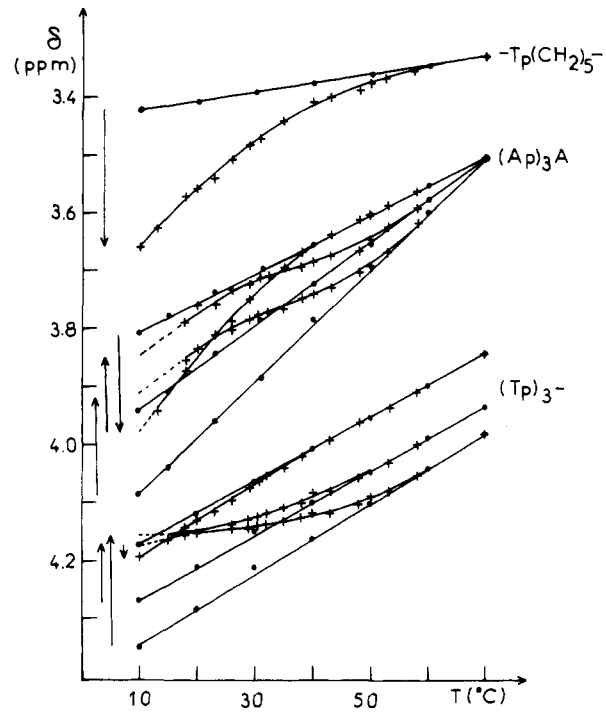

FIGURE 7: Temperature dependence of the ^{31}P resonances in the 1:1 complex $(Ap)_3A$–$(Tp)_4(CH_2)_5$Acr (2.3 mM:2.3 mM). The data for the free molecules are represented by dots (●) and those for the 1:1 mixture by crosses (+). The arrows show the difference in chemical shifts between the uncomplexed and the complexed molecules at 10 °C.

Discussion

The variation of absorbance and chemical shifts vs. temperature has allowed us to determine a temperature of half-transition of 51 °C for a 1:1 mixture of poly(rA) + $(Tp)_4(CH_2)_5$Acr (8 mM:8 mM) and 32 °C for a 1:1 mixture of poly(rA) + $(Tp)_8$Et (10 mM:10 mM). Therefore, the presence of the dye strongly stabilizes the interaction between poly(rA) and the oligothymidylate since an oligonucleotide containing *four* thymines and one acridine derivative exhibits a half-transition temperature that is 17 °C *higher* than that of an *octa*nucleotide without acridine. This conclusion is supported by the fact that $(Tp)_4(CH_2)_5$Acr associates with $(Ap)_3A$ while $(Tp)_4$Et does not under the same experimental conditions.

Binding of $(Tp)_4(CH_2)_5$Acr and $(Tp)_8$Et to poly(rA) induces important upfield shifts of several resonance lines of adenine and thymine. At the temperature of half-transition, the H_2 proton of adenine in poly(rA) is upfield shifted by 0.18 ppm in the presence of $(Tp)_8$Et while it is upfield shifted by 0.38 ppm in the presence of $(Tp)_4(CH_2)_5$Acr. In order to obtain more information on the conformation of the poly(rA)–$(Tp)_4(CH_2)_5$Acr complex, we have compared the upfield shifts corresponding to the formation of this complex to those ob-

served in the poly(rA)–$(Tp)_8Et$ complex for the different protons of adenine and thymine (Table I). Due to the broadening of the resonance lines below 40 °C, the chemical shifts in the complex poly(rA)–$(Tp)_4(CH_2)_5Acr$ were calculated from the relationship

$$\delta_{obsd} = \delta_f \frac{c_f}{c_0} + \delta_b \frac{c_b}{c_0} \quad (1)$$

where δ_f and δ_b are the chemical shifts of the free and complexed molecules, respectively, and c_f and c_b are the respective concentrations of free and bound molecules calculated at each temperature from the melting curve obtained by absorption spectrophotometry at 424 nm on the same sample as that used for ^1H NMR measurements. The comparison of experimental with theoretical values computed with the data of Patel & Tonelli (1975) and Arter & Schmidt (1976) (Table I) clearly shows that the duplex formed between poly(rA) and $(Tp)_8Et$ corresponds to the A form. This conclusion is in agreement with the previously reported formation of an A-type hybrid duplex between oligoribonucleotides and oligodeoxyribonucleotides (Pardi et al., 1981). The binding of $(Tp)_4(CH_2)_5Acr$ to poly(rA) induces a higher upfield shift of the H_2(A) resonance line (0.3 ppm) and of the CH_3(T) resonances (0.1 ppm) but a smaller upfield shift of the H_8(A) resonance as compared with $(Tp)_8Et$. Clearly, the attachment of 2-methoxy-6-chloro-9-aminoacridine to the oligodeoxythymidylate induces some important changes in the structure of the complex. Since complex formation induces an important increase of the line width of both thymines and dye protons, we can conclude that the rotational correlation time of the dye increases strongly with complex formation as also observed for the bases. These results led us to postulate a model of interaction where the dye is intercalated between two consecutive A·T base pairs. Unfortunately, the broadening of the resonance lines of the dye prevented us from obtained information on the chemical shifts of the acridine protons. Additional data could be obtained with shorter molecules, namely, the complex formed by $(Tp)_4(CH_2)_5Acr$ with $(Ap)_3A$ (Table II). The H_8 and especially the H_2 protons of adenine are upfield shifted as well as the CH_3 groups of thymine although the effect is smaller. All the aromatic protons of the acridine dye are also upfield shifted by 0.5–0.8 ppm. These important ring-current effects observed on both adenine and acridine protons show that the aromatic rings of these molecules are closely stacked.

If we assume that magnetic contributions to the relaxation rates of the protons are entirely due to dipole–dipole interactions, the rate for the relaxation of spin I, R_1, due to dipolar interactions with nucleus S is given by eq 2 (Abragam, 1978),

$$R_1 = \frac{\gamma_I^2 \gamma_S^2 \hbar S(S+1)}{12 r^6} [J_0(\omega_1 - \omega_S) + 18J_1(\omega_I) + 9J_2(\omega_1 + \omega_S)] \quad (2)$$

where r is the I–S internuclear separation, γ_I and γ_S are the gyromagnetic ratio of the J and s spins, and $J(\omega)$ is the spectral density at frequency ω resulting from molecular motions that modulate the I–S dipolar interaction. The dependence of spin–lattice relaxation rates upon complex formation between $(Tp)_4(CH_2)_5Acr$ and $(Ap)_3A$ reveals a change in the conformation of the oligonucleotides in the complex as compared with the free molecules. The relaxation rates of the different protons of adenine and thymine are governed by the proximity of different neighboring protons. The distance between the protons governing the dipole–dipole relaxation of protons H_8, H_6, CH_3, and $H_{1'}$ have been computed (Reid et al., 1983). It has been shown that the nearest protons of H_8 are $H_{2'}$ [intraresidue (B form) or interresidue (A form)] and $H_{2''}$ (interresidue, B form). The nearest protons of H_6 are $H_{2'}$ [intraresidue (B form) or interresidue (A form)], $H_{2''}$ (interresidue, B form), and CH_3. The nearest protons of CH_3 are H_6 and $H_{2''}$ (interresidue, B form). The nearest protons of $H_{1'}$ are $H_{2'}$ and $H_{2''}$ (intraresidue).

A change in sugar puckering and in the position of the base relative to the sugar greatly influences the rate of relaxation of the H_8 and H_6 protons. An increase of the relaxation rate can be obtained by an increase of the sum $\sum r^{-6}$ or by an increase of the correlation time. The rate R_1 for the dipolar relaxation of a spin $^1/_2$ induced by another spin $^1/_2$ is given by the relationship

$$R_1 = \frac{3}{10} \frac{\hbar^2 \gamma_4}{r^6} \left[\frac{\tau}{1 + \omega^2 \tau^2} + \frac{4\tau}{1 + 4\omega^2 \tau^2} \right] \quad (3)$$

where τ is the correlation time and ω is the resonance frequency. The knowledge of R_1 at two different frequencies allowed us to compute τ and r. The relaxation times T_1 have been measured at 300 and at 90 MHz (Table III), and the computation of τ and r has been made by assuming that only one proton is participating in the relaxation process of each proton. Data of Table III show that by decreasing the temperature from 45 to 21 °C the correlation times of H_8, $H_{1'}$(T), and $H_{1'}$(A) increase to about 1 ns. The average of internuclear separations $\langle r \rangle$ is only slightly modified upon complex formation (Table III) even though stacking interactions due to A·T base pairs are expected to change the distances between relaxing protons. At 21 °C only part of the complex is formed, which might explain why the observed variations are small.

The broadening of the ^{31}P resonances lines upon complex formation did not bring much information on the conformation of the complex poly(rA)–$(Tp)_4(CH_2)_5Acr$. On the contrary, the investigation of phosphorus resonances in the complex formed by $(Ap)_3A$ with $(Tp)_4(CH_2)_5Acr$ yielded important information. Several factors have an important influence on the ^{31}P chemical shifts in nucleic acids: the ring current of the bases, the ionization state of the phosphate, the changes in the O–P–O angle, and the changes in the phosphate torsional angles. Theoretical and experimental investigations have shown that the major source of variation of chemical shift in oligonucleotides is the passage of a *gg* conformation of the phosphodiester to a *gt* conformation (Gorenstein et al., 1976). The formation of a duplex structure induces an upfield shift by increasing the percentage of the *gg* conformation. Intercalation of a dye in a duplex structure induces a downfield shift by increasing the percentage of the *gt* conformation. Such effects have been observed for example during the binding of oligo(U) to poly(rA) and for the intercalation of ethidium bromide in the duplex poly(rA)·oligo(U) (Goldfield et al., 1983). The important downfield shift of two phosphorus resonance lines of $(Ap)_3A$ (0.21 and 0.13 ppm) strongly suggests than an open *gt* conformation is induced in two phosphodiester linkages upon binding of $(Tp)_4(CH_2)_5Acr$. The upfield shift of the third phosphorus is indicative of an increase in the percentage of *gg* conformation for the third phosphodiester bond. The binding of $(Ap)_3A$ to $(Tp)_4(CH_2)_5Acr$ leads to an increase of the percentage of an open *gt* conformation for two phosphodiester linkages between thymines (downfield shifts of 0.19 and 0.10 ppm) and a strong increase in the percentage of a *gg* conformation for the phosphodiester linked to the aliphatic chain (0.24 ppm). Since an open conformation of phosphodiesters is found twice in $(Ap)_3A$ and in $(Tp)_4$-

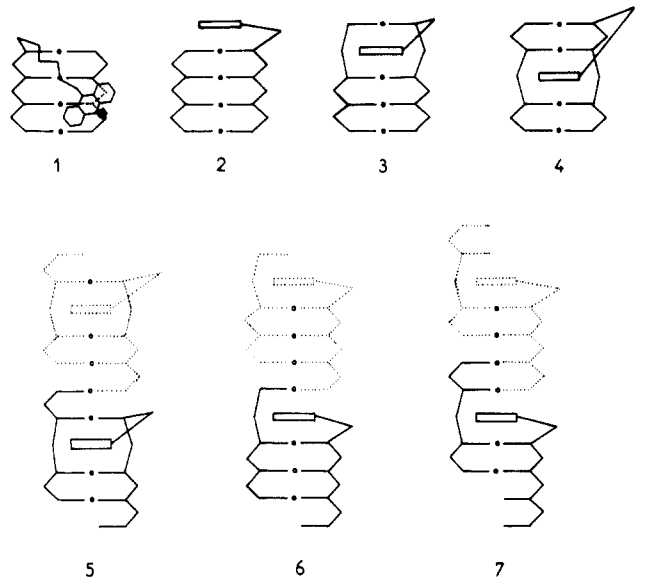

FIGURE 8: Possible schemes of $(Ap)_3A-(Tp)_4(CH_2)_5Acr$ complexes on the basis of CPK models. (1) The acridine and bound by its electrostatic interaction to the last phosphate group of $(Ap)_3A$. (2) The acridine ring is stacked on top of the last thymine–adenine base pair. (3) The acridine ring is stacked between the third and the fourth thymine–adenine base pair. (4) The acridine ring is partially stacked between the second and third thymine–adenine base pair. (5–7) The complex $(Tp)_4(CH_2)_5Acr-(Ap)_3A$ forms less than four base pairs. These complexes can be stabilized by aggregation (dotted lines).

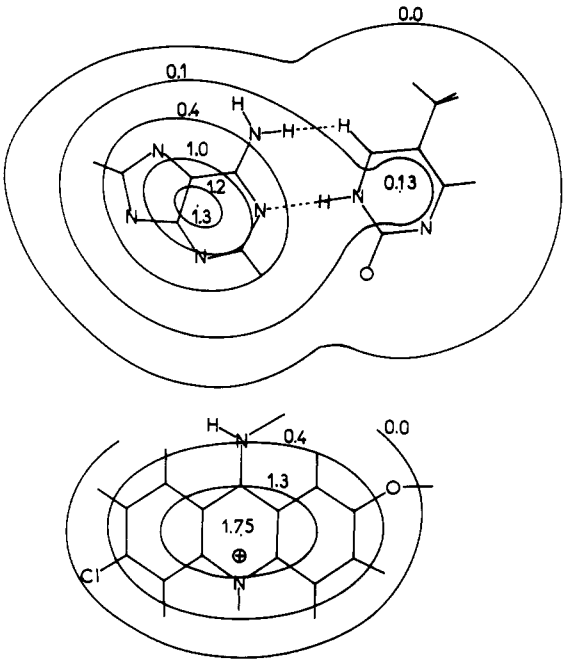

FIGURE 9: Intermolecular shielding values ($\Delta\delta$) due to ring-current effects at 3.4 Å in a plane parallel to an adenine–thymine base pair [after Giessner-Prettre et al. (1976)] and to 2-hydroxy-6-chloro-9-aminoacridine after Giessner-Prettre [see Barbet et al. (1976)].

$(CH_2)_5Acr$, we propose that there exists an equilibrium between several 1:1 complexes.

The downfield shifts of the two phosphorus resonance lines of $(Ap)_3A$ increase when the temperature is lowered from 50 to 30 °C and then remain constant down to 10 °C. This suggests that the equilibrium between the different complex structures is shifted when the temperature is lowered.

Examination of a Corey–Pauling–Koltun (CPK) model of a duplex $(Ap)_3A-(Tp)_4(CH_2)_5Acr$ shows several possible positions of the acridine ring with respect to the duplex structure, which are presented in Figure 8: (1) The acridine ring is outside the mini double helix and is free of any interaction or is electrostatically bound by its NH group to the first or second phosphate of the adenine strand. (2) The acridine ring is stacked on top of the last A·T base pair. (3) The acridine ring is stacked between the third and fourth A·T base pairs. (4) The acridine ring is partially stacked between the second and third A·T base pairs. (5) The duplex forms only three out of four A·T base pairs, and the acridine ring is sandwiched between the last two A·T base pairs. (6 and 7) The duplex is sandwiched between the last A·T base pair and the neighboring adenine. Only three or two base pairs are formed. In the last three models, another molecules of $(Tp)_4(CH_2)_5Acr$ can be associated with the free adenines and an aggregate can be formed with several molecules of $(Ap)_3A$ and $(Tp)_4(CH_2)_5Acr$ overlapping each other (dotted lines in Figure 8).

The complex of type 1 does not lead to open *gt* phosphodiester conformations. This geometry is not in agreement with the ^{31}P and proton NMR data, and we conclude that only a small percentage of this type of complex—if any—can exist in solution. The CPK model shows that the complex of type 3 is obtained without steric hindrance, that the acridine has a strong overlap with base pairs, and that the long axis of the dye is roughly parallel to the long axis of the last but one A·T base pair. Examination of the map of the intermolecular shielding values due to the ring current of 2-methoxy-6-chloro-9-aminoacridine (Figure 9) shows that in the complex of type 3 the H_2 protons of adenines that are nearest to the center of the acridine ring will be more upfield shifted than the H_8 protons of adenine or the H_6 and CH_3 protons of thymine. The insertion of the acridine ring leads to the formation of one open *gt* conformation of a phosphodiester bond both in $(Ap)_3A$ and in $(Tp)_4(CH_2)_5Acr$. Although the exact geometry of the hybrid complex is not known (mixture of $C_{3'}$ endo and $C_{2'}$ endo conformation of the sugar), the effects induced by this type of complex are in agreement with the NMR data. All the other types of complexes induce a stacking of the dye with one or two A·T base pairs (with the long axis of acridine roughly parallel to the long axis of one A·T base pair in type 2 and type 5 and perpendicular in type 4) and the formation of one open *gt* conformation between phosphodiester bonds linking two adenines. Complexes of type 5 and 7 are of special interest since they represent the only structures involving an open *gt* conformation of a phosphodiester bond in $(Ap)_3A$, which is different from that in the complex of type 3 or 6. From a thermodynamic point of view, complex 3 is certainly the most stable with the acridine ring intercalated between two A·T base pairs and four A·T base pairs formed between the complementary oligonucleotides. From model building and from the geometry at the intercalation site, complex 4 does not correspond to a very favorable configuration. Stacking of the acridine dye on top of the four A·T base pairs as in complex 2 is more favorable even though this structure cannot account for the ^{31}P NMR results. Complexes 5–7, which form three or two A·T base pairs, are certainly less stable than the complex of type 3, which forms four A·T base pairs, but they may be stabilized by aggregation as shown in Figure 8 (dotted lines). Using the values of the relaxation time T_1 obtained at 90 and 300 MHz, we can compute a correlation time of the H_8 protons of adenines in the complex $(Tp)_4-(CH_2)_5Acr-(Ap)_3A$, which is of the order of 1 ns at 21 °C. This correlation time corresponds to the rotation of a sphere whose radius is about 10 Å or to a DNA rod 10 Å long (Broersma, 1960). This result is not compatible with the

formation of long aggregates of complexes 5–7 for which the rotational correlation time should be much longer. Any aggregate of type 7 will have two unpaired adenines at one end and two unpaired thymines at the other end. This will not be very favorable if the aggregate contains only a few units. Below 15 °C the line width of the proton resonances increases and shows evidence for intermediate chemical exchange at 300 MHz. We can only use the line width of the phosphorus resonances at 121.5 MHz (12 Hz at 10 °C and 1 Hz at 21 °C) to estimate the correlation time of the complex. This value can be compared to the line width observed on short DNA duplexes where the relaxation processes are of the same type as those in the complex $(Tp)_4(CH_2)_5Acr-(Ap)_3A$. For samples of DNA containing 140 base pairs, Hogan & Jardetsky (1980) have reported a line width of 22 Hz at 40.5 MHz at 23 °C. Shindo (1980) has reported a line width of 28 Hz at 40.3 MHz and of 103 Hz at 109.3 MHz at 19 °C. We have measured a line width of 2 Hz for the phosphorus resonances of the duplex formed by the self-complementary decanucleotide d(AATTGCAATT) at 22 °C (unpublished results). The data indicate that the complexes of $(Tp)_4(CH_2)_5Acr-(Ap)_3A$ form aggregates at low temperature and that these aggregates involve more than 10 and less than 140 base pairs. This estimate can be refined by the computation of the line width of the phosphorus resonance as a function of the correlation time. Shindo (1980) has published such a relationship for nucleic acids, taking into account the contributions of both chemical shift anisotropy and dipole–dipole interactions. Using this relationship, we have computed a correlation time of the order of 10^{-8} to 5×10^{-8} s, which correspond to a number of base pairs in the range 20–40. It seems therefore reasonable to conclude that the most likely structures for $(Tp)_4(CH_2)_5Acr-(Ap)_3A$ complexes are 2 and 3 above 20 °C and that structures 5–7 are obtained below 20 °C. Further experiments using different base sequences that will not permit sliding of one oligonucleotide with respect to the other (such as in complexes 5–7) should help determine which of the structures presented in Figure 7 is the most likely to form.

ACKNOWLEDGMENTS

We thank Dr. J. Y. Lallemand (Paris) for allowing us to make use of the Bruker WM 400 spectrometer.

Registry No. $(Tp)_4(CH_2)_5Acr$, 91401-15-9; $(Tp)_4Et$, 95647-07-7; $(Tp)_8Et$, 95647-08-8; poly(rA), 24937-83-5; $(Ap)_3A$, 4042-12-0; adenine, 73-24-5; thymine, 65-71-4.

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