Hydrogen Bonding in Deoxyribonucleic Acid Base Recognition. 1. Proton Nuclear Magnetic Resonance Studies of Dinucleotide–Acridine Alkylamide Complexes†

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ABSTRACT: For studies on the possible involvement of hydrogen bonding in base recognition from the outside of the nucleic acid double helix, 2-methoxy-6-chloro-9-aminoacridine derivatives bearing a carboxamide side chain were examined by ^{1}H NMR spectroscopy. The study of the interaction of these derivatives with CpG or GpC demonstrated that (i) the 2-methoxy-6-chloro-9-aminoacridine ring intercalates preferentially in the minihelix formed by CpG, which indicates a relative pyrimidine-(3′–5′)-purine sequence specificity that contrasts with the simple 9-aminoacridine ring wherein Reuben et al. [Reuben, J., Baker, B. M., & Kallenbach, N. R. (1978) *Biochemistry 17*, 2916–2919] did not observe any sequence preference, (ii) the geometry of the intercalated minihelical complex of the 2-methoxy-6-chloro-9-[(5-carbamoylpentyl)-amino]acridine with CpG as deduced from isoshielding curves resembles that found in the crystalline complexes of proflavin, with several autocomplementary dinucleoside monophosphates, (iii) the terminal carboxamide group borne by the side chain of 2-methoxy-6-chloro-9-[(5-carbamoylpentyl)amino]acridine (**5**) intercalated in CpG lies in the small groove and seems to interact through hydrogen bonds with the adjacent guanine.

The selective recognition of DNA sequence by proteins (repressor, RNA polymerase) probably involves specific interactions of amino acid side chains with nucleic acid bases. Seeman et al. (1976) and Hélène (1977) have emphasized the capacity of the carboxamide groups of asparagine and glutamine to form two hydrogen bonds with either AT or GC base pairs in the large groove or the minor groove, respectively, without modifying the normal Watson–Crick structure. Moreover, aromatic amino acids could be involved in base recognition processes through the intercalation of their aromatic moiety (Dimicoli & Hélène, 1974a,b).

Specific hydrogen bonding between the 2-amino group of guanine and the peptide side chains of actinomycin D has been implicated in the guanine specificity of this antibiotic (Jain & Sobell, 1972). Therefore actinomycin might be regarded as a very simple model of DNA–protein binding since both intercalation of its aromatic part and specific contacts between its amino acid component and base pairs through hydrogen bonding occur as assumed for DNA–protein complexes (Seeman et al., 1976; Hélène, 1977).

On the other hand, many DNA binding ligands [hydroxystilbamidine (Festy & Daune, 1973), tilorone hydrochloride (Chandra & Woltersdorf, 1976)] and DNA binding antibiotics [quinomycin and Triostin (Lee & Waring, 1978) and actinomycin D (Müller & Crothers, 1968; Sobell, 1973)] show base specificity. Several of these compounds are DNA intercalating molecules and have clinical applications. Consequently in search of new antitumoral compounds, we have thus far synthetized bisintercalating compounds which are characterized by very strong DNA binding affinities (Barbet et al., 1975; Le Pecq et al., 1975; Roques et al., 1976; Delbarre et al., 1977; Gaugain et al., 1978a,b). Some of these compounds have already shown high antitumor properties (Roques et al., 1979). Now, it would of course be very interesting to obtain molecules having not only high binding affinities but sequence specificity as well.

Therefore, molecules made up of an intercalating ring bearing a side chain with a carboxamide group able to form hydrogen bonds were synthesized. Such molecules were expected to intercalate their aromatic rings in DNA, and the carboxamide group located at the end of the side chain would form specific hydrogen bonds with base provided that the length and the conformation of this side chain were appropriate. The acridine ring was chosen as the intercalating chromophore because of its well-known fluorescent and intercalating properties. When the acridine ring of such molecules is intercalated in DNA, it is easy to see on space-filling models that in order for two hydrogen bonds to form between the terminal carboxamide group of the side chain and one of the two adjacent base pairs (with either guanine in the small groove, or adenine in the large groove) the length of the flexible alkyl chain must be at least five carbons long.

The first step was to study the interaction of these derivatives with RNA minihelices using ^{1}H NMR spectroscopy to try to get direct evidence of both their intercalation and the involvement of hydrogen bonding in their selective recognition of certain bases. Results of these studies are reported in the present paper.

The second step was to study the interaction of a series of analogues of these derivatives with DNA in order to verify that the results obtained at the minihelix level could be valided at the macromolecular level. Results of these DNA studies are reported in the accompanying paper (Markovits et al., 1981).

Materials and Methods

The acridine compounds **2**, **4**, **5**, and **6** listed in Figure 1 were synthesized as described in the following paper (Mar-

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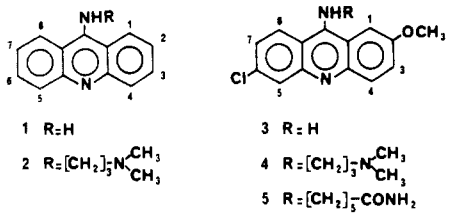

FIGURE 1: Structural formulas of the 9-aminoacridines **1** and **2** and the 2-methoxy-6-chloro-9-aminoacridine derivatives **3**–**5**.

kovits et al., 1981). Compounds **1** and **3** were purchased from Aldrich. The ribodinucleosides monophosphates CpG, GpC, and UpA were obtained from Sigma Chemical Co. and used without further purification.

Proton magnetic resonance spectra were recorded at 270 MHz with a Bruker WH 270 spectrometer operating in the Fourier transform mode and locked to the deuterium resonance of the solvent D_2O. When experiments were performed in H_2O solutions, locking was done on the deuterium resonance of the buffer (CD_3COONa). The water resonance was reduced by standard homonuclear gated decoupling as well in D_2O as in H_2O solution. Probe temperatures were regulated to ±1 °C by a Bruker B ST 100/700 controller and monitored by observing the splitting in ethylene glycol.

Stock solutions of the dinucleosides monophosphates and the acridine derivatives were prepared in 0.04 M sodium deuterioacetate buffer, pH 5.6, containing disodium ethylenediaminetetraacetate (10^{-4} M). pH values were determined by direct pH meter reading, without correction, on a Tacussel TC 60/N pH meter, equipped with a combined capillary electrode. Chemical shifts were measured with respect to an internal reference, sodium 3-(trimethylsilyl)propionate-*2,2,3,3-d*$_4$ (Merck), and are reliable to ±0.01 ppm. The line widths at half-height of the methylene signals of the chain were determined according to Casy (1971).

Models of intercalating complexes were constructed by superimposing scale drawings of acridine, guanine, and cytosine rings. The DNA base pairs were drawn along with the isoshift lines computed by Giessner-Prettre & Pullman (1976a); they involve both ring current and atomic anisotropy contributions at a distance of 3.4 Å perpendicular to the base planes. The acridine geometry was taken from crystallographic data (Courseille et al., 1977). Theoretical proton shifts $\Delta\delta_c$ of the acridine were estimated for a fixed orientation of the dye molecule relative to the bases. Linear interpolation between the isoshielding curves of the bases was used to obtain theoretical shift with an estimated error of less than 15% the given shift value.

The self-association constants (K) were computed by using the equation of Dimicoli & Hélène (1973) under the assumption that the acridine derivatives self-associate to form vertically stacked n-mers without cooperativity as already reported (Barbet et al., 1976). Let B_0 be the total concentration of the dye. A plot of $(\Delta\delta/B_0)^{1/2}$ vs. $\Delta\delta$ for each proton gives a straight line with slope $s = [K/(2\Delta\delta B_2)]^{1/2}$. Each line intercepts the x axis at $x_0 = 2\Delta\delta B_2$, allowing a chemical shift difference between free molecule and stacked dimer ($\Delta\delta B_2$) to be obtained for every proton. Furthermore, the association constant was easily calculated ($K = x_0 s^2$) for each proton. The reported constants K correspond to average values.

Results

In all the experiments, the NMR spectra of the studied acridine derivatives were assigned by selective irradiation as already described (Barbet et al., 1976).

(*a*) *Self-Association of Acridine Derivatives.* The NMR study of the interactions between intercalating agents and complementary ribodinucleoside monophosphates requires the knowledge of the chemical shifts which correspond to free intercalating compounds. However, these molecules tend to self-associate in water at the concentration used for the NMR study of the intercalation process. Consequently a preliminary study of the self-stacking of the acridine derivatives had to be performed following the variation of the chemical shifts of the aromatic moiety as a function of the acridine concentration from 2×10^{-3} M to 5×10^{-5} M in D_2O and 0.04 M sodium deuterioacetate buffer, pH 5.6, at 25 °C. When their concentrations are lowered, a large deshielding effect is observed for all the aromatic protons of most of the acridine derivatives. 2-Methoxy-6-chloro-9-aminoacridine (**3**) exhibits the strongest effects (Figure 1Sa, supplementary material), and a plot of $(\Delta\delta/B_0)^{1/2}$ vs. $\Delta\delta$ gives straight lines for each aromatic protons (Figure 1Sb; see paragraph at end of paper regarding supplementary material), yielding a high self-association constant (3300 M^{-1}). Likewise, Reuben et al. (1978) observed an important self-stacking of the 9-aminoacridine **1**. Interestingly enough 9-[[3-(dimethylamino)propyl]amino]acridine (**2**) does not exhibit significant chemical shifts variation within the concentration range studied here (2×10^{-3} to 5×10^{-5} M). Therefore it can be concluded that compound **2** weakly self-associates. The repulsion between the positively charged alkylamino chains of **2** at our experimental pH (pH 5.6) might account for its lower self-association tendency. Consequently neither the association constant of **2** nor the chemical shifts differences ($\Delta\delta B_2$) between free molecule and stacked dimer could be determined.

In contrast, in the series of 2-methoxy-6-chloro-9-aminoacridine, the large deshielding effect observed during the dilution for all the aromatic protons of every molecule allows us to accurately determine ($r > 0.99$) δ_0, the chemical shifts at infinite dilution, $\Delta\delta B_2$, the chemical shift difference between the free molecule and the stacked dimer, and K, the association constant. All the self-association parameters are reported in Table 1S (supplementary material). The association constants are respectively 3300, 107, and 1300 M^{-1} for compounds **3**, **4**, and **5**. The following must be noticed:

(i) The extrapolated chemical shifts of the aromatic protons at zero concentration are similar for all three compounds, **3**–**5**. Such results indicate an absence of strong electronic effects on the aromatic moiety brought about by the different substituent introduced on C-9.

(ii) The self-association constant greatly depends on the nature of the side chain attached at the 9 position. The association constant of acridine **4** (with an alkylamino chain) and **5** (with a pentylcarbamoyl chain) are lowered 33-fold and 2.5-fold, respectively, in relation to the compound without a side chain, **3**. The additional repulsion caused by the charged amino groups in **4** can well account for the destabilization of aggregates in that case.

(iii) The difference in the chemical shifts of the aromatic protons between the free molecule and the stacked dimer ($\Delta\delta B_2$) are much larger in the parent compound, **3** than in the substituted derivatives **4** and **5**. This result might well indicate that the acridine chromophores are more closely stacked in the case of the parent compound **3** than in compounds **4** and **5**. This could be related to the lack of steric hindrance and/or electrostatic repulsion in the absence of side chain.

(iv) The comparison of self-association of compounds **2** and **4** shows that the stacking between acridine rings is increased by the presence of the chloro and methoxy groups. The in-

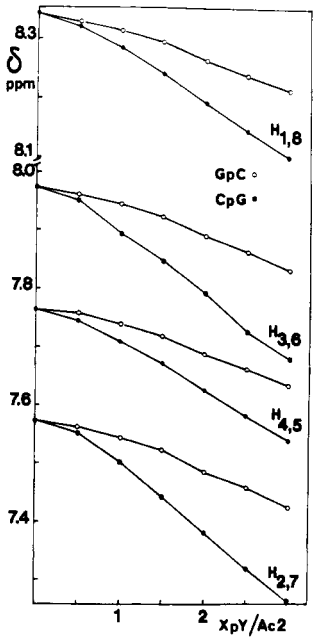

FIGURE 2: Titration of 9-[[3-(dimethylamino)propyl]amino]acridine (**2**) with ribodinucleoside monophosphates CpG and GpC. Dye concentration is 2 mM in D_2O–0.04 M sodium deuterioacetate buffer, pH 5.6; T 25 °C.

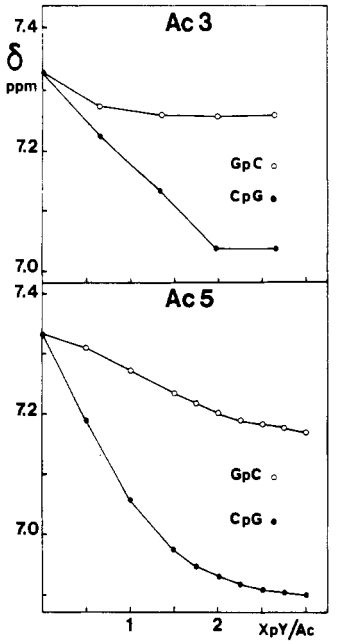

FIGURE 3: Titration of the H_7 resonance of Ac3 [= 2-methoxy-6-chloro-9-aminoacridine (**3**)], 1.5 mM, and of Ac5 [= 2-methoxy-6-chloro-9-[(5-carbamoylpentyl)amino]acridine (**5**)], 2 mM, with ribodinucleoside monophosphates CpG and GpC in D_2O–0.04 M sodium deuterioacetate buffer, pH 5.6; T 25 °C.

creased polarizability of the acridine ring induced by these substituents might explain the tendency of these 2-methoxy-6-chloro-9-aminoacridines to self-associate as well as their higher DNA binding affinities as compared to the unsubstituted 9-aminoacridine (J. B. Le Pecq and B. P. Roques, unpublished results).

(*b*) *Geometry of the Self-Stacked Complexes of the 2-Methoxy-6-chloro-9-aminoacridines* **3**–**5**. Since compounds **4** and **5** exhibit the same chemical shift differences ($\Delta\delta B_2$) between the free state and stacked dimers (Table 1S, supplementary material), it clearly appears that both acridine derivatives self-associate in the same manner, with one chromophore inverted with respect to the other as previously described for compound **4** (Barbet et al., 1976). Such a geometry seems likely because it minimizes the repulsive electrostatic interactions between the charged nitrogen on the side chain and on the ring by keeping them away from each other (Delbarre et al., 1976). Although larger values of $\Delta\delta B_2$ are observed in the case of **3**, the same stacking geometry can be also suggested for this compound since the ratio of $\Delta\delta B_2$ of **3** to the $\Delta\delta B_2$ of **4** (or **5**) is constant for all protons.

(*c*) *Base-Pair Sequence Specificity*. Various studies in solution as well as in solid state have shown that several intercalating compounds exhibit a clear sequence specificity at the oligonucleotide level [for a review, see Krugh & Nuss (1979)]. On the other hand, theoretical calculations (Ornstein & Rein, 1979a,b; Broyde & Hingerty, 1979) predict that it is easier to unstack the pyrimidine-(3′–5′)-purine sequence than it is the purine-(3′–5′)-pyrimidine sequence. Accordingly, if free energy for unstacking a base pair is a limiting factor of intercalation, these calculations predict that intercalation will occur preferentially in the pyrimidine-(3′–5′)-purine sequence (Ornstein & Rein, 1979a,b; Broyde & Hingerty, 1979; Nuss et al., 1979).

Nevertheless it was observed that daunorubicine, actinomine (Krugh et al., 1979), and 9-aminoacridine (Reuben et al., 1978) show no clear sequence preference and that actinomycin D interacts preferentially with GpC, a purine–pyrimidine sequence. Thus it appears that the nature of the intercalating ring as well as the presence of a side chain (actinomine vs. actinomycin) could influence the sequence specificity of intercalating agents. Therefore, the interactions of different acridine derivatives with autocomplementary ribodinucleoside monophosphates were studied by ^{1}H NMR.

The upfield shifts of the aromatic protons of **2** with CpG and with GpC are reported in Figure 2. The titration curves indicate a relative preference for the pyrimidine-(3′–5′)-purine sequence. Such a preference is not observed with the 9-aminoacridine **1** which is devoid of a side chain (Reuben et al., 1978). This result brings to mind the difference in sequence specificity between actinomine and actinomycin. However, none of the titration curves of **2** show a break followed by a plateau corresponding to the formation of complexes of a definite stoichiometry. Nevertheless, when a sample with 3 equiv of GpC and 1 equiv of **2** is allowed to stand overnight at 25 °C, it yields a yellow crystalline complex. Its stoichiometry has been determined in Me_2SO and is found to equal a 2:1 ratio of GpC:acridine derivative.

The pyrimidine-(3′–5′)-purine sequence specificity of the three 2-methoxy-6-chloro-9-aminoacridine derivatives **3**–**5** was therefore studied comparatively. The ^{1}H NMR spectra were recorded for each compound with increasing amounts of either CpG or GpC. Results for compounds **3** and **5** are reported in Figures 2S, 3S, 3, and 4 (Figures 2S and 3S are in the supplementary material). Results for compound **4** are not shown, but they are similar to those obtained with compound **5**, except that titration curves do not present so well defined a plateau, indicating a weaker affinity than for compound **5**.

All the aromatic protons and the methoxy group of **5** (Figure 3S, supplementary material) are strongly upfield shifted upon complex formation with CpG, and the titration curves present a break for the 2:1 ribodinucleoside monophosphate to drug ratio as shown for the H_7 resonance (Figure 3). Such behavior characterizes the formation of a 2:1 complex where one acridine ring has intercalated into the miniature double helix formed by the two hydrogen-bonded dinucleoside monophosphates (Krugh & Reinhardt, 1975). It should be noticed that the formation of such a complex is induced by the intercalating agent, since at 21 °C and in the range of CpG

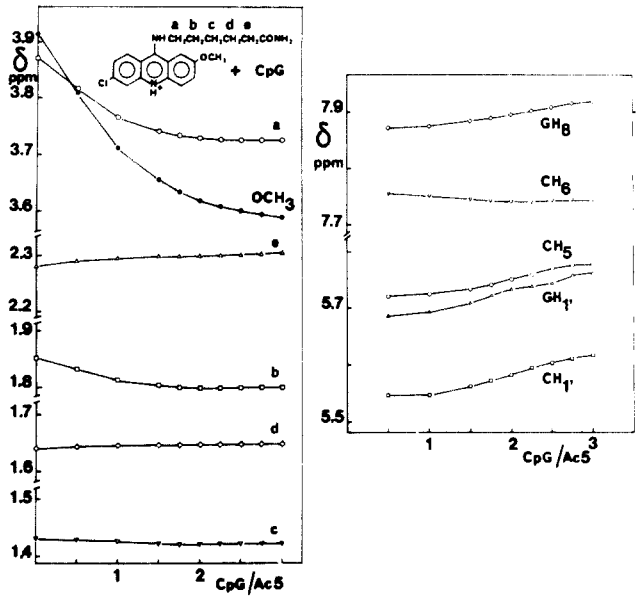

FIGURE 4: Titration of 2-methoxy-6-chloro-9-[(5-carbamoylpentyl)amino]acridine (**5**) (2 mM) with CpG, in D_2O–0.04 M sodium deuterioacetate buffer, pH 5.6; T 25 °C. (a) Variation of the chemical shifts of the methylene and methoxy groups of **5**. (b) Variation of the chemical shifts of the guanine and cytosine protons and of the ribose $H_{1'}$ protons upon CpG–5 complex formation.

concentration used in these experiments (10^{-3} M to 6×10^{-3} M) the formation of the Watson–Crick pairing does not occur unless there is an intercalating compound (Krugh et al., 1976).

Moreover, the methylene groups of the side chain are affected differently by the complexation of the acridine. These features indicate a preferential orientation of the side chain which will be later discussed. Finally, the formation of a 2:1 CpG:**5** complex is also supported by a significant shielding of the cytidine H_5 and $H_{1'}$ and the guanine H_8 and $H_{1'}$ protons at low CpG:acridine ratio (Figure 4). This effect is due to the strong current shift of the intercalated acridine ring. In contrast, the interaction of **5** with GpC under the same conditions leads to a weaker upfield shift for all protons (Figure 3S, supplementary material).

The variations in the chemical shifts of the aromatic protons of compound **3** (2-methoxy-6-chloro-9-aminoacridine without a side chain) for increasing concentrations of CpG and GpC are reported in Figures 2S (supplementary material) and 3 for the H_7 resonance. The interpretation of these titration curves is made difficult by the strong self-association of this acridine compound. Nevertheless the shielding effect induced on OCH_3, H_7, and H_5 protons clearly indicates a preference for CpG binding as opposed to GpC and furthermore demonstrates that the affinity of this compound for the CpG minihelix is much larger than its self-association constant ($K = 3.3 \times 10^3$ M^{-1}). Finally, the formation of intercalated complexes can be illustrated by the determination of the induced chemical shifts $\Delta\delta_i$ (Table I) which correspond to the difference between δ_0, the chemical shifts for the free dye (extrapolated at zero concentration), and δ, the chemical shifts of the intercalated molecule.

These titration experiments lead us to note the following: (i) The introduction of the chloro and methoxy groups enhances the binding of the 9-aminoacridine ring to the minihelices and promotes pyrimidine–purine sequence specificity, in accordance with an enhanced polarizability of the heterocycle. (ii) The introduction of side chains on the 2-methoxy-6-chloro-9-aminoacridine does not modify the pyrimidine-(3′–5′)-purine vs. purine-(3′–5)-pyrimidine sequence

Table I: Upfield Shifts, $\Delta\delta_i$ (ppm), of 9-Amino- and 2-Methoxy-6-chloro-9-aminoacridine Derivatives **2–5** Induced by Intercalation with Ribodinucleoside Monophosphates CpG and GpC[a]

	2		3		4		5	
	CpG	GpC	CpG	GpC	CpG	GpC	CpG	GpC
H_1	0.28	0.18	1.00	0.93	0.55	0.38	0.79	0.58
H_2	0.27	0.13						
H_3	0.26	0.13	0.71	0.47	0.51	0.31	0.64	0.36
H_4	0.27	0.18	0.76	0.66	0.44	0.29	0.60	0.43
H_5	0.27	0.18	0.87	0.73	0.60	0.37	0.77	0.54
H_6	0.26	0.13						
H_7	0.27	0.13	0.68	0.45	0.49	0.30	0.60	0.32
H_8	0.28	0.18	0.75	0.67	0.43	0.28	0.66	0.48
OCH_3			0.49	0.15	0.32	0.19	0.41	0.21

[a] $\Delta\delta_i = \delta_{2.5} - \delta_0$ is the difference between the chemical shift, $\delta_{2.5}$, in the 2.5:1 ribodinucleoside monophosphate:dye complex and the chemical shift, δ_0, of the dye, extrapolated at zero concentration.

preference which is in contrast to what is observed with the simple 9-aminoacridine (Reuben et al., 1978). (iii) Under our conditions, none of the acridines interacts significantly with UpA, as was already reported for several intercalating agents (Krugh & Reinhardt, 1975; Davidson et al., 1977). (iv) Only the (carbamoylpentyl)acridine **5** exhibits a saturation process corresponding to a 2:1 stoichiometry for CpG. Note that in this complex only one phosphate group was neutralized by the protonated acridine. This minihelical complex remains soluble at a high nucleotide to drug ratio whereas in the corresponding CpG complexes with the bis positively charged (aminoalkyl)acridine **2** and **4**, neutral precipitates occur at the 2:1 ratio.

Finally, in order to compare the intercalating ability of acridine at the oligonucleotide level in similar conditions as those used for DNA studies (Markovits et al., 1981, following paper), the ionic strength was increased by the addition of NaCl from 0.03 M to 1.5 M in the 3:1 CpG:**5** complex at pH 5.6. The absence of significant variations in the chemical shifts and the ribose $^3J_{H_1'H_2'}$ coupling constants until 1.0 M Na^+ shows that the complex remains stable even at high ionic strength. However, further addition of NaCl leads to the sudden precipitation of acridine.

(*d*) *Geometries of Acridines at the Intercalation Site.* The shielding effects observed for the aromatic protons of all these molecules are average values of the shielding experienced by the complexed molecule stacked into the miniature double helix formed by two dinucleoside monophosphates and the shielding of the free molecule arising from its self-stacking. However if the binding affinity of the dye for the ribodinucleoside monophosphates is large enough and if there is only one preferred geometry for the complex, a sharp break in the titration curves is observed at a characteristic dinucleoside to drug ratio. Beyond this ratio, the chemical shifts of the acridine, which no longer vary significantly, are considered to be equal to the chemical shift of the complexed form alone. The induced shieldings $\Delta\delta_i$ due to the intercalation of the acridine into the minihelix are summarized in Table I. From these results it clearly appears that stronger shieldings are obtained in the series of the 2-methoxy-6-chloro-substituted 9-aminoacridine as compared to the unsubstituted 9-aminoacridine **1** (Reuben et al., 1978) or to the 9-[[3-(dimethylamino)propyl]amino]acridine, **2**. On the other hand, study of the titration curves of acridines **2–5** show that only the 2-methoxy-6-chloro-9-aminoacridine (**3**) and its (carbamoylpentyl)amino derivative **5** exhibit breaks at a 2:1 dinucleoside to drug ratio. Moreover, in the case of compound

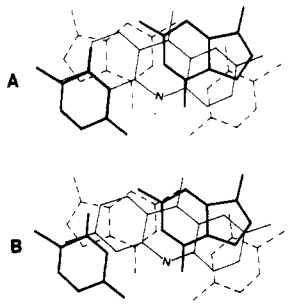

FIGURE 5: Possible geometries of intercalation for compounds 3 (model A) and 5 (model B) with CpG, deduced from comparison of computed, $\Delta\delta_c$, and experimental, $\Delta\delta_i$, upfield shifts reported in Tables I and IIS (supplementary material).

5, chemical shifts in the 2:1 complexes remain almost constant when the temperature is lowered from 25 to 15 °C. Therefore, although shielding effects have been measured for all these acridines, only those corresponding to compounds 3 and 5 can be validly used for the determination of the geometry of the intercalating complexes. Such geometries were estimated by use of the ring currents and atomic anisotropy effects of the different bases of the DNA and acridine ring previously computed by Giessner-Prettre & Pullman (1976a,b).

In the absence of any crystallographic data about intercalated complex with compounds of the 2-methoxy-6-chloroacridine series, a geometry of the intercalation of compounds 3 and 5 in CpG minihelix must take into account the shielding effects reported in Table I. These data show the following:

(i) All the aromatic protons of the acridine 3 and 5 are strongly shielded in their intercalated complexes. Moreover the magnitude of these strong shielding effects requires both a position almost symetrical with respect to the plane of the upper and lower base pairs and a sliding of these latter upon the acridine rings with a concomitant increase in the base turn angle.

(ii) H_1 is the most upfield shifted of all the aromatic protons, and the methylene group CH_2(a) of compound 5 experiences a shielding of up to 0.45 ppm. These results can only be explained if the 9-amino group of the acridines lies in the minor groove of the minihelices, in which case the methylene group CH_2(a) as well as the aromatic protons H_1 and H_8 will be located near the upper and lower guanine rings of the hydrogen-bonded base pairs and undergo the stronger shielding effects of these aromatic residues.

The induced shielding $\Delta\delta_c$ of the acridine protons in several intercalating geometries determined from crystallographic studies on minihelices [for a review, see Tsai (1978)] have been computed from isoshielding curves and compared with the experimental $\Delta\delta_i$ (Table 2S, supplementary material). From these comparisons, the best fits correspond to the two geometries shown in Figure 5 which are characterized by the same large base turn angle (B = 29°). These two geometries are very close to those found in the minihelical complexes of proflavin with the autocomplementary ribodinucleoside monophosphates ICpG, B = 36° (Reddy et al., 1979), and CpG, B = 32° (Neidle et al., 1977). Nevertheless, the introduction of the carbamoylalkyl chain on the 9-amino group of the 2-methoxy-6-chloro-9-aminoacridine leads to small sliding of the two base pairs with respect to the acridine ring (Figure 5, model B). This could be related to either a larger steric hindrance conferred by the carbamoylalkyl chain or to the formation of hydrogen bonds between the carboxamide group and the guanine.

(*e*) *Involvement of the Carboxamide Group of 2-Methoxy-6-chloro-9-[(5-carbamoylpentyl)amino]acridine* (5) *in*

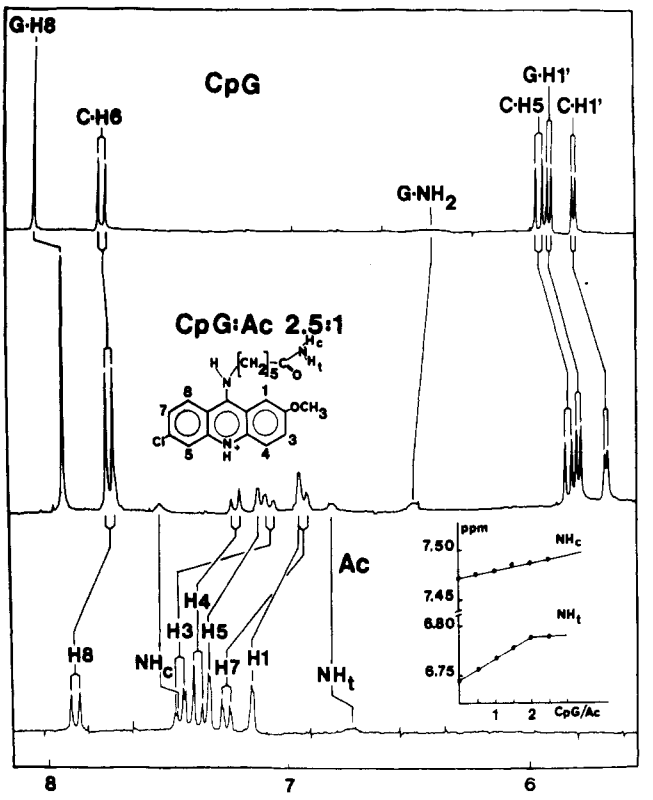

FIGURE 6: 270 MHz ^{1}H NMR spectra of CpG (top), CpG:2-methoxy-6-chloro-9-[(5-carbamoylpentyl)amino]acridine 2.5:1 complex (middle), and 2-methoxy-6-chloro-9-[(5-carbamoylpentyl)amino]-acridine (5) (bottom). Acridine concentration is equal to 10^{-2} M; spectra were run in H_2O–0.4 M sodium deuterioacetate buffer, pH 5.6. Inset = titration curves of the trans and cis NH amide protons with CpG in the above conditions.

Hydrogen Bonding with Guanine in the CpG–5 Complex. Evidence of the formation of hydrogen bonds between the carboxamide group of 5 and guanine in the CpG–5 minihelical complex derives from three kinds of measurements.

(*i*) *NMR Studies of the Interaction of* 5 *with CpG in* H_2O. The first indication of a strong interaction between CpG and 2-methoxy-6-chloro-9-[(5-carbamoylpentyl)amino]acridine (5) came from the observation that CpG (2×10^{-2} M) was able to solubilize the acridine 5 at a concentration (10^{-2} M) and a temperature (15 °C) where 5 alone usually precipitated. Such behavior had already been observed with actinomycin and dpGpC (Patel, 1974). However, the titration experiments were performed at 40 °C in order to obtain the spectrum of 5 without CpG. As shown in Figure 6 (bottom), the restricted rotation around the C–N amide bond leads to separate resonances for the two NH protons in the spectra of 5. In accordance with Bovey (1969), the signal at high field was assigned to the trans amide proton (NH_t), i.e., cis to the carbonyl group. It is important to note that at 40 °C the ribodinucleoside monophosphates (Figure 6, top) exist only as monomers in the absence of intercalating agent (Krugh et al., 1976). Despite these conditions (40 °C, 10^{-2} M), formation of the minihelix nucleated around the acridine 5 is again demonstrated by the large shifts of the aromatic protons (Figure 6, middle), which, although smaller, are similar to those observed at 25 °C with the 2×10^{-3} M sample in D_2O as described above.

Moreover, the incremental addition of CpG to the (carbamoylpentyl)acridine 5 solution induces a much larger deshielding for the amide proton signal located at high field (NH_t) than for the cis one. On the other hand, the titration curve for the NH_t tends to level off at a 2:1 dinucleotide to

drug ratio which corresponds to the stoichiometry of the complex (Figure 6, inset).

As shown in Figure 6, it appears clearly that the complexation process leads to a narrowing in the line widths of the GNH_2 and amide protons. This effect corresponds to a decrease of their exchange rates which probably results from hydrogen bonding in the intercalated complex. However, the deshieldings of these exchangeable resonances are much smaller than those observed in a netropsin–octanucleotide complex (Patel, 1979). Such differences could result from several points: (a) The CpG:**5** complexation was performed at 40 °C whereas the netropsin–octanucleotide one was studied at 20 °C. (b) At these two temperatures, the netropsin–octanucleotide complex is obviously much more stable than the CpG:**5** intercalated minihelix. (c) The exchangeable protons undergo the shielding effect of the acridine ring (NH_t 0.20–0.30 ppm in the proposed geometry; Figure 5B); this effect does not exist in the nonintercalated netropsin–octanucleotide complex. Therefore the chemical shifts of the GNH_2 and amide protons in the CpG–**5** complex result from opposite effects leading to low downfield shifts.

Further evidence of the formation of a 2:1 CpG:acridine **5** minihelical complex stabilized by additional hydrogen bonds was obtained following the variation of the chemical shifts of the amide exchangeable protons as a function of the temperature. In the free dye the two amide protons of **5** exhibit similar temperature dependencies with slopes of 6.7×10^{-3} ppm/°C, but the formation of the minihelical complex (2.5:1 CpG:**5**) only affects the NH_t amide proton by decreasing its slope to 5.1×10^{-3} ppm/°C.

(ii) Restricted Motion of the Acridine Side Chain. The line width of the CH_2 groups is dependent upon both the overall molecular reorientation of the complex and the internal segmental motion of the chain which reflects the degree of freedom of each methylene. Therefore, the involvement of the alkyl chain in the hydrogen-bonding scheme can be followed by examination of the line width of the methylene groups during the breakdown of the intercalated complex induced by a temperature increase. In the free acridine **5**, line widths of these methylene vary regularly within a very narrow range as a function of the temperature (results not shown). In contrast, in the 2.5:1 CpG:**5** complex, the line widths of these CH_2 are affected differently as a function of their position when the temperature rises from 20 to 70 °C (Figure 7). The regular decrease in the line width of CH_2(b) reflects the dissociation of the complex whereas the faster narrowing of the CH_2(d) and CH_2(e) in the 20–40 °C temperature range could be due to the breakdown of the hydrogen bonds between the amide group and the guanine which precedes the fraying of the minihelix. This interpretation is reinforced by the behavior of the CH_2(c), located in the middle of the chain, whose line width remains practically temperature independent. This melting process can be followed from the classical sigmoidal curves corresponding to the deshielding of the aromatic protons as a function of the temperature. In the 2.5:1 CpG:**5** complex this feature is shown in Figure 7 for the H_3 acridine proton and leads to a fraying curve with a midpoint around 45 °C.

(iii) Chemical Shift of Side-Chain Protons in the CpG–5 Complex. The methylene groups of the side chain are affected differently by the complexation of the acridine (Figure 4). The CH_2(b) is clearly shielded whereas the CH_2(e) is slightly deshielded. These opposite effects could be interpreted by the perpendicular orientation of the side chain to the acridine ring with the amide group directed toward the guanine ring. In such a geometry, the CH_2(a) and CH_2(b) experience shielding

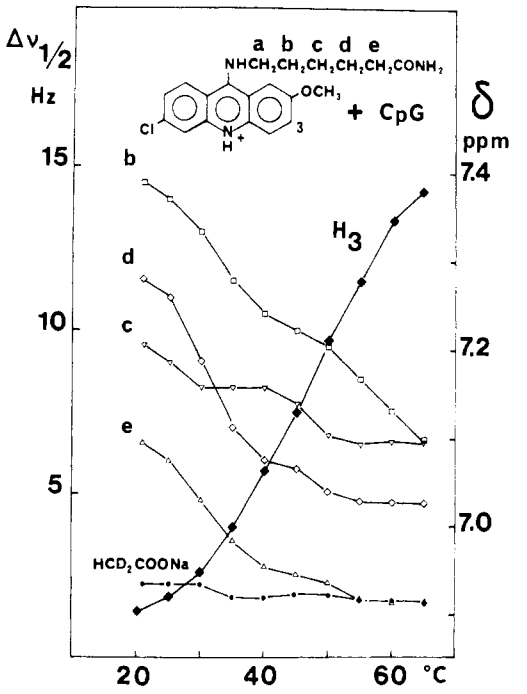

FIGURE 7: Variation of the line width at half-height of the methylene groups and variation of the chemical shift of H_3 in the 2.5:1 CpG:2-methoxy-6-chloro-9-[(5-carbamoylpentyl)amino]acridine complex as a function of the temperature in 0.4 M sodium deuterioacetate buffer, pH 5.6, with 10^{-2} M acridine **5**.

current shifts from the superimposed base pairs while the CH_2(e) group experiences a deshielding due to its position in the plane of the base pairs.

Furthermore, shielding of the corresponding CH_2(b) group in the chain of **4**, which equally forms a stronger complex with CpG but without additional hydrogen bonds, does not appear (results not shown).

All these results give strong evidence that the carboxamide group is immobilized at the level of the plane of the guanine ring and forms a hydrogen bond with this base.

Conclusion

The use of ^1H NMR in D_2O and H_2O allowed us to follow the formation of intercalated minihelices of new acridines derivatives with the ribodinucleoside monophosphates CpG and GpC and the formation of hydrogen bonds between the amide group on the alkyl chain of 2-methoxy-6-chloro-9-[(5-carbamoylpentyl)amino]acridine (**5**) and the guanine ring of the self-associated ribodinucleoside monophosphate CpG.

These measurements enable us to obtain information relative to two kinds of problems: first, the base-pair sequence specificity of intercalating compounds; second, the possibility of base recognition through hydrogen bonding from the outside of the DNA helix.

(a) Sequence Specificity of Intercalating Agents. Theoretical calculations predict that all intercalating molecules would prefer the pyrimidine-(3′–5′)-purine sequence in the absence of specific base interaction (Ornstein & Rein, 1979a,b, and references cited therein; Broyde & Hingerty, 1979). Our results agree with this prediction. Nevertheless, the absence of pyrimidine-(3′–5′)-purine sequence specificity of 9-aminoacridine reported by Reuben et al. (1978) and the appearance of such specificity when the 9-aminoacridine bears a charged amino chain (compound **2**) or when 2-methoxy and 6-chloro groups are introduced on the 9-aminoacridine ring (compound **3**) remain puzzling. It is interesting to note that this difference of specificity between 9-aminoacridine and 2-methoxy-6-

chloro-9-aminoacridine derivatives for pyrimidine-(3′–5′)-purine sequences corresponds to different binding for the intercalating dimers made up by these two different rings. Thus, dimers composed of two 9-aminoacridines can intercalate their two rings between adjacent base pairs (Wakelin et al., 1978) while corresponding dimers composed of two 2-methoxy-6-chloro-9-aminoacridines can only intercalate their two rings with two base pairs between them (Le Pecq et al., 1975). This behavior might be related to a sequence specificity effect. In the first case, if binding is independent of sequence, the two rings may intercalate in two adjacent sites of different sequence. In the second case, if binding is dependent on pyrimidine-(3′–5′)-purine sequence, one ring will intercalate between base pairs of pyrimidine-(3′–5′)-purine sequence while the second one will not be able to intercalate in one of the two adjacent sites which obviously cannot be a pyrimidine-(3′–5′)-purine sequence.

However, it should be noted that the introduction of an alkyl chain, also present in the dimers, on the 9-aminoacridine ring (compound **2**) leads again to a pyrimidine-(3′–5′)-purine sequence specificity. Therefore the differences in the binding process shown by the two kinds of dimers may be related to the size of their intercalating rings. Indeed the pyrimidine-(3′–5′)-purine sequence has a higher flexibility, especially in the ϕ and χ angles, than the purine-(3′–5′)-pyrimidine one (Broyde & Hingerty, 1979). Consequently, due to its lower degree of freedom, this latter sequence may not be able to adjust large intercalating moieties such as the 2-methoxy-6-chloro-9-aminoacridine ring, hindering intercalation of the second acridine chromophore in the nearest-neighbor site in accordance with the excluded site model (Crothers, 1968). In contrast, due to its smaller size, the 9-aminoacridine derivatives could be free to search for the energy-minimized configuration in the pyrimidine-(3′–5′)-purine as well as in the purine-(3′–5′)-pyrimidine sequences, in which case the two rings could intercalate in two adjacent sites. This assumed relative mobility of the 9-aminoacridine in the intercalating site is in accordance with the presence of two kinds of intercalated minihelices in the crystal of the 9-aminoacridine–ICpG complex (Sakore et al., 1979) and with the absence of clear stoichiometry in the titration curves of **2** with CpG or GpC which is shown in this paper.

Therefore at the polymer level the bisintercalation of dimers might depend, among other factors, on sequence specificity effects and structural alterations of the DNA helix.

(*b*) *Guanine Recognition by Hydrogen Bonds from the Outside of the Helix.* Studies of the complex between the mini double helix formed by CpG and the 2-methoxy-6-chloro-9-[(5-carbamoylpentyl)amino]acridine (**5**) by ^{1}H NMR spectroscopy strongly suggest that the carboxamide group of the intercalated acridine side chain is involved in hydrogen bonding in the complex. The main observations were that the acridine side chain and, more important, its terminal methylene group (e) were immobilized in the complex, the trans NH amide of the carboxamide group was more deshielded than the cis NH in the complex, and its temperature dependence decreased upon interaction with CpG. Guanine is very probably the base involved in the hydrogen bonding because the side chain, as already mentioned, lies in the small groove. Under this condition, the 2-amino group and the N3 of guanine are the only two available sites for hydrogen bonding with a carboxamide group, as has already been discussed by Seeman et al. (1976) and Hélène (1977). Additional arguments in support of this conclusion will be presented in the accompanying paper.

Supplementary Material Available

Self-association parameters for derivatives **2**–**5** (Table 1S), comparison between computed upfield shifts, $\Delta\delta_c$, and experimental upfield shifts, $\Delta\delta_i$, for compounds **3** and **5** in their complex with CpG (Table 2S), concentration dependency of the proton chemical shifts of derivative **3** (Figure 1Sa), self-association of derivative **3** obtained from curves of Figure 1Sa (Figure 1Sb), titration of 2-methoxy-6-chloro-9-aminoacridine (**3**) with CpG and GpC (Figure 2S), and titration of 2-methoxy-6-chloro-9-[(5-carbamoylpentyl)amino]acridine (**5**) with CpG and GpC (Figure 3S) (5 pages). Ordering information is given on any current masthead page.

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Hydrogen Bonding in Deoxyribonucleic Acid Base Recognition. 2. Deoxyribonucleic Acid Binding Studies of Acridine Alkylamides†

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ABSTRACT: A series of derivatives of 2-methoxy-6-chloro-9-aminoacridine bearing side chains terminated by various groups, such as carboxamide, *N*-methylcarboxamide, *N,N*-dimethylcarboxamide, methyl ester, and methyl were synthesized. The interaction of these intercalating dyes with DNA of various GC content was studied comparatively by using equilibrium dialysis and fluorescence spectroscopy. The results showed that the compounds bearing a chain terminated by a free carboxamide group could interact specifically with GC-rich DNA provided that the length and conformation of the side chain were appropriate. From these results in hand as well as those reported in the preceding paper (NMR studies at the minihelix level) it is thought that the G specificity of these compounds arises from their ability to form hydrogen bonds between the terminal carboxamide group borne by their side chain and the NH_2 and N3 of the adjacent guanine located in the small groove.

In the preceding paper (Gaugain et al., 1981), the interaction of acridine derivatives bearing a carboxamide group on a side chain (acridine alkylamide) with ribodinucleoside monophosphates was studied by using ^1H NMR spectroscopy. The purpose of this study, as has already been mentioned, was to investigate the possible involvement of hydrogen bonding in the specific recognition of DNA sequence by a variety of ligands. It was observed at this minihelix level that indeed the carboxamide group of acridine pentylamide formed hydrogen bonds with the guanine base. It then became necessary to transpose these studies to the polynucleotide level. However, with polymers, NMR studies cannot be used to directly demonstrate the formation of hydrogen bonding. Consequently we have been led to adopt an indirect approach. A variety of 2-methoxy-6-chloroacridines bearing alkyl chains of various length ended with potentially bisinteracting groups such as $CONH_2$ and $CONHCH_3$, potentially monointeracting groups such as $CON(CH_3)_2$ and $COOCH_3$, or noninteracting groups such as CH_3 were synthesized. The 2-methoxy-6-chloro-9-aminoacridine ring was selected because of its well-known fluorescent and intercalating properties and its absence of base specificity (Weisblum & deHaseth, 1972; Pachmann & Rigler, 1972; Arndt-Jovin et al., 1979).

Examination of space-filling models shows that if the acridine ring is intercalated and if a flexible alkyl chain is used to connect the 9-amino group of the acridine ring and the carboxamide group, then a chain length of 4 to 6 carbon atoms will allow both the carbonyl and the NH_2 group of the terminal amide group to form hydrogen bonds with a base pair immediately above or below the site of intercalation. Therefore,

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