Markovits, J., Gaugain, B., Barbet, J., Roques, B. P., & Le Pecq, J. B. (1981) *Biochemistry* (following paper in this issue).
Müller, W., & Crothers, D. M. (1968) *J. Mol. Biol. 35*, 251–290.
Neidle, S., Achari, A., Taylor, G. L., Berman, H. M., Carrell, H. L., Glusker, J. P., & Stallings, W. C. (1977) *Nature (London) 269*, 304–307.
Nuss, M. E., Marsh, F. J., & Kollman, P. A. (1979) *J. Am. Chem. Soc. 101*, 825–833.
Ornstein, R. L., & Rein, R. (1979a) *Biopolymers 18*, 1277–1291.
Ornstein, R. L., & Rein, R. (1979b) *Biopolymers 18*, 2821–2847.
Patel, D. J. (1974) *Biochemistry 13*, 2388–2395.
Patel, D. J. (1979) *Eur. J. Biochem. 99*, 369–378.
Reddy, B. S., Seshadri, T. P., Sakore, T. D., & Sobell, H. M. (1979) *J. Mol. Biol. 135*, 787–812.
Reuben, J., Baker, B. M., & Kallenbach, N. R. (1978) *Biochemistry 17*, 2916–2919.
Roques, B. P., Barbet, J. B., Oberlin, R., & Le Pecq, J. B. (1976) *C.R. Hebd. Seances Acad. Sci., Ser. D 283*, 1365–1367.
Roques, B. P., Pelaprat, D., Le Guen, I., Porcher, G., Gosse, C., & Le Pecq, J. B. (1979) *Biochem. Pharmacol. 28*, 1811–1815.
Sakore, T. D., Reddy, B. S., & Sobell, H. M. (1979) *J. Mol. Biol. 135*, 763–785.
Seeman, N. C., Rosenberg, J. M., & Rich, A. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 804–808.
Sobell, H. M. (1973) *Prog. Nucleic Acid Res. Mol. Biol. 13*, 153–190.
Tasi, C. C. (1978) *Annu. Rep. Med. Chem. 13 (32)*, 316–326.
Wakelin, L. P. G., Romanos, M., Chen, T. K., Glaubiger, D., Canellakis, E. S., & Waring, M. J. (1978) *Biochemistry 17*, 5057–5063.

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

Judith Markovits,‡ Bernard Gaugain,§ Jacques Barbet,‖ Bernard P. Roques,§ and Jean-Bernard Le Pecq*,‡

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,

† From the Laboratoire de Physico-Chimie Macromoléculaire, Institut Gustave-Roussy, 94800 Villejuif, France, and the Département de Chimie Organique, Université René Descartes, 4, avenue de l'Observatoire, 75006 Paris, France. *Received July 10, 1980; revised manuscript received January 20, 1981.* Support of this research through grants from Université Pierre et Marie Curie (Paris VI), Université René Descartes (Paris V), CNRS, INSERM 140, INSERM SCN 21, the Délégation à la Recherche Scientifique et Technique, and the Petroleum Research Fund, administered by the American Chemical Society, is gratefully acknowledged.

‡ Present address: Laboratoire de Physico-Chimie Macromoléculaire (LA No. 147 du CNRS et U 140 de l'INSERM), Institut Gustave-Roussy, 94800 Villejuif, France.
§ Present address: Département de Chimie Organique (ERA No. 613 du CNRS), UER des Sciences Pharmaceutiques et Biologiques, 4, avenue de l'Observatoire, 75270 Paris Cedex 06, France.
‖ Present address: Centre d'Immunologie de Marseille-Luminy, INSERM-CNRS Case 906, 13288 Marseille Cedex 2, France.

0006-2960/81/0420-3042$01.25/0

the specificity of binding of these different derivatives for GC-rich DNA has been compared as a function of their chain length and the nature of the terminal group.

The relationship between the preference of these acridine derivatives for GC-rich DNA and the proper spatial location of the hydrogen-bonding group support the argument that hydrogen bonding occurs in the DNA polymer as well as in the dinucleotide.

Experimental Procedures

Materials

(*1*) *Synthesis*. The synthesis of the various acridine derivatives is reported in the supplementary material (see paragraph at end of paper regarding supplementary material).

(*2*) *DNA and Oligonucleotides*. DNAs from *Clostridium perfringens*, *Micrococcus luteus*, and calf thymus (Sigma) were purified by three phenol extractions.

Poly[d(A-T)]·poly[d(A-T)], poly[d(G-C)]·poly[d(G-C)], and poly[d(I-C)]·poly[d(I-C)] (Boehringer) were used without further purification.

Molar DNA concentrations were determined by using the following extinction coefficients expressed per nucleotide at 260 nm: *Clostridium perfringens* 6230 M^{-1} cm^{-1}, *Micrococcus luteus* 6900 M^{-1} cm^{-1}, and calf thymus 6400 M^{-1} cm^{-1} (Müller & Crothers, 1975); poly[d(A-T)]·poly[d(A-T)] 6800 M^{-1} cm^{-1}, poly[d(I-C)]·poly[d(I-C)] 6900 M^{-1} cm^{-1} at 251 nm, and poly[d(G-C)]·poly[d(G-C)] 8400 M^{-1} cm^{-1} at 254 nm according to Wells et al. (1970).

Methods

(*A*) *Determination of Base Specificity. Equilibrium Dialysis Experiments*. The determinations were made according to Müller & Crothers (1975).

Equilibrium dialysis was performed in two steps by using three-chamber Teflon dialysis cells (Oriel, Paris). In the first step the outer chambers (1 and 2) are filled with 0.8 mL of DNA_1 and DNA_2 solutions, respectively, in equimolar nucleotide concentrations (3.2×10^{-4} M). The inner chamber (3) is filled with the solution of acridine derivative (0.8 mL) whose concentrations range from 5×10^{-7} to 10^{-4} M. All the solutions are made in 0.05 M sodium cacodylate buffer, pH 6, with 0.4 M NaCl. The filled dialysis cells are equilibrated for 24 h in an agitated thermostatic bath (22 °C). In the second step, the already equilibrated solutions are withdrawn from the three compartments. The three chambers are then washed with sodium cacodylate buffer (Na^+ 0.45 M; pH 6), after which they are refilled with the same solutions as in step one and equilibrated for 48 h as described above. Finally the dye concentrations are determined by spectrofluorometry (λ_{exc} 420 nm; λ_{em} 500 nm) after 1:1 dilution with 0.5% acetic acid in dimethyl sulfoxide according to Müller et al. (1973) in order to liberate the acridine from complexes.

Fluorescence measurements were made with a photon counting spectrofluorometer build in the laboratory (Paoletti, 1972). Since only the outer compartments contain DNA, at equilibrium the outer compartments will contain bound plus free dye while the central compartment will contain only free dye.

If C_1 and C_2 are the total dye concentrations measured in the outer compartments 1 and 2, respectively, and if C_3 is the free dye concentration measured in the central compartment, then

$$\alpha' = (C_1 - C_3)/(C_2 - C_3) \quad (1)$$

where α' is the ratio of the concentrations of DNA_1-bound dye to DNA_2-bound dye.

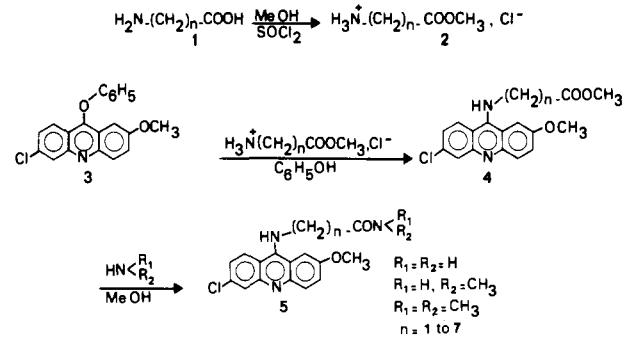

FIGURE 1: Scheme for the synthesis of the 2-methoxy-6-chloro-9-[(ω-carbamoylalkyl)amino]acridines.

According to Müller & Crothers (1975), α is defined as $\lim_{r \to 0} \alpha'$, where r is the number of dye bound per base pair.

Fluorometric Assay of Base Specificity. This assay is based on the fact that the fluorescence of these 2-methoxy-6-chloro-9-aminoacridine derivatives is almost totally quenched when they come into contact with one GC base pair. Thus, when these compounds bind to DNA, their fluorescence quantum yield is highly dependent on the DNA base composition.

A small concentration (10^{-6} M) of 2-methoxy-6-chloro-9-(alkylamino)acridine derivative is added to a concentrated solution (2×10^{-4} M) of DNA with a different base composition. The continuous variation of DNA base composition was obtained by mixing two DNAs of different AT content (DNAs of *Micrococcus luteus* and *Clostridium perfringens*). The fluorescence intensity of this solution (I) was measured. Likewise, the fluorescence intensities (I_{AT} and I_{GC}) of solutions containing the same amount of dye (10^{-6} M) with 2×10^{-4} M poly[d(A-T)]·poly[d(A-T)] (I_{AT}) or 2×10^{-4} M poly[d-(G-C)]·poly[(dG-C)] (I_{GC}) were measured and the ratio $(I - I_{GC})/(I_{AT} - I_{GC})$ was computed.

The standard conditions for these measurements are fluorescence excitation at 474 nm and fluorescence emission at 500 nm.

(*B*) *Viscosimetry*. Viscometric measurements were performed at 25 °C as described earlier (Saucier et al., 1971; Revet et al., 1971; Le Pecq et al., 1975).

Results

(*1*) *Synthesis*. All the alkylcarbamoyl derivatives of 2-methoxy-6-chloro-9-aminoacridine are prepared according to the scheme outlined in Figure 1. (Details of the syntheses are given in the supplementary material.) The characteristics of all the synthetized compounds are summarized in Table I.

(*2*) *Studies of DNA Base Specificity*. (*a*) *DNA Intercalation Ability*. Because it had been reported (Ramstein et al., 1972; Ramstein & Leng, 1975) that proflavin tended not to intercalate in GC-rich DNA, it was necessary to check that our 2-methoxy-6-chloro-9-[(ω-carbamoylalkyl)amino]acridine could intercalate into DNA, regardless of the DNA base composition. For several derivatives the DNA length increase upon binding was measured by viscosimetry (Saucier et al., 1971) using DNAs of various GC content. The observed DNA length increase was independent of the nature of DNA and very close to that measured for ethidium bromide (results not shown).

(*b*) *Equilibrium Dialysis Experiments*. The Müller & Crothers procedure (1975) was used to determine the preference of each drug in this study for GC-rich and AT-rich DNAs. Specifically, we determined the values of α

$$\alpha = \lim_{r \to 0} \alpha' = r_{DNA_1}/r_{DNA_2} \quad (2)$$

Table I: Analytical and Specificity Data of a Series of 2-Methoxy-6-chloro-9-(alkylamino)acridine Derivatives[a] $Ac(CH_2)_nR$

compound	n	R	mp (°C)	mol formula	equilibrium dialysis experiments: α	K_2/K_1 (model 1)	K_2/K_1 (model 2)	fluorometric assay: K_2/K_1 (model 1)	K_2/K_1 (model 2)
1	1	$COOCH_3$	>270 (Cl^-)	$C_{17}H_{15}N_2O_3Cl$	nd			2.5	3
2	2	$COOCH_3$	>270 (Cl^-)	$C_{18}H_{17}N_2O_3Cl$	1.45	>20	5	4.4	5
3	5	$COOCH_3$	80	$C_{21}H_{23}N_2O_3Cl$	1.16	2.2	1.6	3	3.5
4	1	$CONH_2$	230	$C_{16}H_{14}N_3O_2Cl$	nd			3	3.5
5	2	$CONH_2$	204	$C_{17}H_{16}N_3O_2Cl$	1.58	>20	>12	10	15
6	3	$CONH_2$	202	$C_{18}H_{18}N_3O_2Cl$	1.12	1.2	1.4	3.7	4
7	4	$CONH_2$	200	$C_{19}H_{20}N_3O_2Cl$	1.05	1.3	1.2	2	2
8	5	$CONH_2$	182	$C_{20}H_{22}N_3O_2Cl$	1.41	>20	4.4	6.5	8
					4.5*	4.5*	4.5*		
9	6	$CONH_2$	180	$C_{21}H_{24}N_3O_2Cl$	1.37	>20	3.8	6	6.5
10	7	$CONH_2$	177	$C_{22}H_{26}N_3O_2Cl$	1.35	>10	3.5	5	5.5
11	5	$CONHCH_3$	168	$C_{21}H_{24}N_3O_2Cl$	1.43	>20	4.8	6	7
12	5	$CON(CH_3)_2$	>270 (CH_3COO^-)	$C_{22}H_{26}N_3O_2Cl$	1.15	2	1.6	2.5	3
13	5	NH_2	190–191	$C_{19}H_{22}N_3OCl$	1.11	1.7	1.4	2.2	2.5
14	5	CH_3	107–108	$C_{20}H_{23}N_2OCl$	1.02	1.05	1.1	1.3	1.4

[a] The microanalyses are in good agreement with the calculated values (C, ±0.26%; H, ±0.75%; N, ±0.6%; O ±0.2%; Cl ±1.5%). α is defined in the text by eq 2. For compound **8**, the asterisk refers to the value computed from binding measurements with poly[d(G-C)]·poly[d(G-C)] vs. poly[d(I-C)]·poly[d(I-C)]. K_2/K_1, ratio of the binding affinity for preferred sites to the binding affinity for nonpreferred sites, is computed for model 1 from eq 6 and 7 and for model 2 from eq 8 and 9.

where r_{DNA_1} and r_{DNA_2} are the number of drug molecules bound per base pair measured for two DNAs with different GC content and equilibrated with the same free dye concentration.

The results of these α measurements using calf thymus DNA (58% AT) and *Micrococcus luteus* DNA (28% AT) are shown in Table I. These results clearly indicate that the acridine derivative **14**, with a simple alkyl side chain, elicits no binding specificity. On the other hand, a clear preference for GC-rich DNA is displayed by several other acridine derivatives, especially some of those whose side chain bears a terminal carboxamide group (compounds **5** and **8** in Table I).

As discussed by Müller & Crothers (1975), the quantitative interpretation of these results, that is, the evaluation of the relative binding affinities for the preferred and nonpreferred sites, requires us to determine the nature of the preferred sites. At low values of r, we can consider the different types of sites to be independent. There are 16 different dinucleotide sequences but there are only 10 different intercalation sites because, at the level of the double-stranded structure, two dinucleotides of complementary sequences lead to a single type of structure. For instance, the two different dinucleotide sequences ApA and TpT give only one type of intercalation site

$$\left|\begin{matrix}(T:A)\\(T:A)\end{matrix}\right|$$

but this site, which can be constructed on two different ways, occurs with a frequency twice as large as that of sequences like

$$\left|\begin{matrix}(T:A)\\(A:T)\end{matrix}\right|$$

which can only be constructed in one way.

An intercalation site which can be constructed in only one way occurs with a frequency of $^1/_{16}$ while an intercalation site which can be constructed in two ways occurs with a frequency of $^1/_8$. Therefore, there are three different intercalation sites with the purine–pyrimidine sequence, three with the pyrimidine–purine sequence, and four with the purine–purine or pyrimidine–pyrimidine sequence.

The equilibrium dialysis experiments can therefore be described by the following equations. Let r_i be the number of dyes bound at the ith site occurring with the frequency f_i, K_i be the corresponding binding affinity, and c_f be the free dye concentrations. At low r values

$$r_i = K_i c_f f_i \tag{3}$$

$$\alpha = \frac{\sum_{i=1}^{10} (K_i f_i)_1}{\sum_{i=1}^{10} (K_i f_i)_2} \tag{4}$$

where $(K_i f_i)_1$ and $(K_i f_i)_2$ refer respectively to the DNAs present in the outer chambers 1 and 2 of the dialysis cell.

(*c*) *Fluorometric Experiments.* The intercalation of the 2-methoxy-6-chloro-9-aminoacridine ring upon contact with a GC base pair leads to an almost complete quenching of its fluorescence (Weisblum & deHaseth, 1972; Pachmann & Rigler, 1972). Therefore the ratio $(I - I_{GC})/(I_{AT} - I_{GC})$, determined as described under Experimental Procedures, measures the fraction of dye bound to the three intercalation sites containing two AT pairs

$$\left|\begin{matrix}(A:T)\\(A:T)\end{matrix}\right| \quad \left|\begin{matrix}(T:A)\\(A:T)\end{matrix}\right| \quad \left|\begin{matrix}(A:T)\\(T:A)\end{matrix}\right|$$

This relation is only valid for low values of r where the energy transfer between different bound acridine cannot occur.

Let i refer to these three sites (i = 1, 2, 3). Therefore:

$$\frac{I - I_{GC}}{I_{AT} - I_{GC}} = \frac{\sum_{i=1}^{3} K_i f_i}{\sum_{i=1}^{10} K_i f_i} \tag{5}$$

(*d*) *DNA Base-Pair Specificity.* Since only two independent measurements are performed, the preceding equations can only be solved by considering at most three groups of sites with three different binding constants, K_1, K_2, and K_3. In this case, the two ratios K_3/K_1 and K_2/K_1 can be obtained and K_1, K_2, and K_3 are the average of the binding constants of the different sites included in each group and weighted according to their respective frequency.

In their study, Müller & Crothers (1975) only performed equilibrium dialysis experiments, and they were limited to the determination of a ratio, K_2/K_1, which refers to only two groups of sites. In addition, they only considered base pair

sequences, in which case there could only consider four different kinds of sites:

site 1 $\begin{vmatrix}(A:T)\\(A:T)\end{vmatrix}$ frequency p^2

site 2 $\begin{vmatrix}(G:C)\\(A:T)\end{vmatrix}$ frequency $p(1-p)$

site 3 $\begin{vmatrix}(A:T)\\(G:C)\end{vmatrix}$ frequency $p(1-p)$

site 4 $\begin{vmatrix}(G:C)\\(G:C)\end{vmatrix}$ frequency $(1-p)^2$

if p is the fraction of of AT base pairs.

Writing a base pair in parentheses means that it can be taken in either direction; for instance, (AT) is either T-A or A-T. Therefore each group of sites includes several sites of different base sequences. The binding constant for a group of sites is then an average value, which is weighted according to the respective frequencies of the different base sequence sites composing the group. Müller and Crothers examined the three different possible models for GC specific ligands.

Model 1. A single GC base pair is sufficient to confer the specificity. Therefore sites 2, 3, and 4 are equivalent. If K_1 refers to site 1 and K_2 refers to sites 2 + 3 + 4, from eq 4 and 5 we obtain

$$\alpha = \frac{(K_1/K_2)p_1^2 + (1 - p_1^2)}{(K_1/K_2)p_2^2 + (1 - p_2^2)} \tag{6}$$

$$\frac{I - I_{GC}}{I_{AT} - I_{GC}} = \frac{(K_1/K_2)p^2}{(K_1/K_2)p^2 + (1 - p^2)} \tag{7}$$

since the frequency of (AT) − (AT) sequence is p^2 and the frequency of sites 2 + 3 + 4 is $(1 - p^2)$, p being the fraction of AT base pair in DNA.

Model 2. Müller & Crothers (1975) assume in that case that the G specificity is obtained only when the GC base pair is either below or above the intercalated dye. Therefore, in this model, there are two equivalent ways of grouping the four different sites, (1 + 2 and 3 + 4) or (1 + 3 and 2 + 4).

If K_1 is the binding constant for the group of sites containing site 1 and K_2 the binding constant for the group of sites containing site 4, from eq 4 and 5 we obtain

$$\alpha = \frac{(K_1/K_2)p_1 + (1 - p_1)}{(K_1/K_2)p_2 + (1 - p_2)} \tag{8}$$

$$\frac{I - I_{GC}}{I_{AT} - I_{GC}} = \frac{(K_1/K_2)p^2}{(K_1/K_2)p + (1 - p)} \tag{9}$$

Model 3. According to this model two adjacent GC base pairs are necessary to confer the specificity of binding. The two groups of sites are therefore site 1 + 2 + 3 with binding constant K_1 and site 4 with binding constant K_2. In this case, from eq 4 and 5 we obtain

$$\alpha = \frac{(K_1/K_2)p_1(2 - p_1) + (1 - p_1)^2}{(K_1/K_2)p_2(2 - p_2) + (1 - p_2)^2} \tag{10}$$

$$\frac{I - I_{GC}}{I_{AT} - I_{GC}} = \frac{(K_1/K_2)p^2}{(K_1/K_2)p^2 + (K_1/K_2)2p(1 - p) + (1 - p)^2} \tag{11}$$

In Table II we present the theoretical values of α computed for the three models and for different values of K_2/K_1.

For each compound the experimental value of α (Table I) can hence be compared to the theoretical value of α according to each of the three models (Table II). For instance, the 2-methoxy-6-chloro-9-[(5-carbamoylpentyl)amino]acridine derivative **8** will elicit an almost complete specificity for the GC base pair in the first model but will only elicit a respectively 5- or 2.5-fold greater affinity for its preferred site in the second and third model.

Table II: Theoretical Values of α Computed for Several Ratios K_2/K_1 [a]

K_2/K_1	α model 1	α model 2	α model 3
1	1	1	1
2	1.15	1.21	1.29
5	1.28	1.45	1.8
7	1.31	1.51	2
10	1.33	1.56	2.19
15	1.35	1.61	2.38
20	1.36	1.63	2.49
40	1.37	1.67	2.69
100	1.38	1.7	2.83
∞	1.39	1.71	2.92

[a] K_2 and K_1 are respectively the binding affinities for preferred and for nonpreferred sites using eq 6 (model 1), 8 (model 2), and 10 (model 3) with $p_1 = 28\%$ and $p_2 = 58\%$, the corresponding AT content of DNA_1 (*Micrococcus luteus*) and of DNA_2 (calf thymus) used in the equilibrium dialysis experiments.

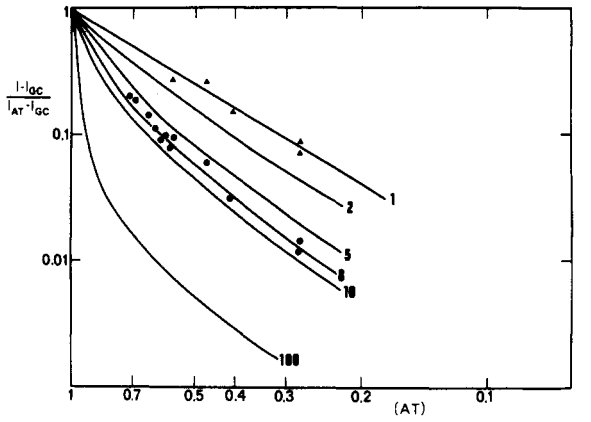

FIGURE 2: Comparison between fluorescence ratios $(I - I_{GC})/(I_{AT} - I_{GC})$ measured for 2-methoxy-6-chloro-9-[(5-carbamoylpentyl)-amino]acridine (**8**, ●) and 2-methoxy-6-chloro-9-(hexylamino)acridine (**14**, ▲) as a function of DNA AT content. The same ratios are computed for different values of K_2/K_1 (affinity for preferred sites/affinity for nonpreferred sites) by using eq 9 according to model 2 (log–log) representation. The ratios $(I - I_{GC})/(I_{AT} - I_{GC})$ are measured as described under Experimental Procedures.

The DNA binding of one of the most specific derivatives, **8**, has been further analyzed. The Scatchard plots deduced from equilibrium dialysis measurements with *Micrococcus luteus* and *Clostridium perfringens* DNAs were obtained. Both Scatchard plots are smoothly curved (results not shown), but the slope obtained with *Micrococcus luteus* DNA is on the average 5 to 10 times larger than the slope obtained with *Clostridium perfringens* DNA. These results are not consistent with model 1 which implies a very large binding constant for a limited number of sites.

Likewise, the apparent values of K_2/K_1 can be obtained in the three different models using the fluorometric assay. To illustrate this possibility, in Figure 2 $(I - I_{GC})/(I_{AT} - I_{GC})$ has been plotted as a function of p on a log–log scale (p being the fractional AT content of the DNA). These data are compared to the theoretical function computed for several values of K_2/K_1 from eq 9 (model 2). The best fit among the experimental data and the computed ones allows us to estimate the apparent value of K_2/K_1 for different compounds. In Figure 3 we compare the values of K_2/K_1 obtained for the three different models by these process using either equilibrium

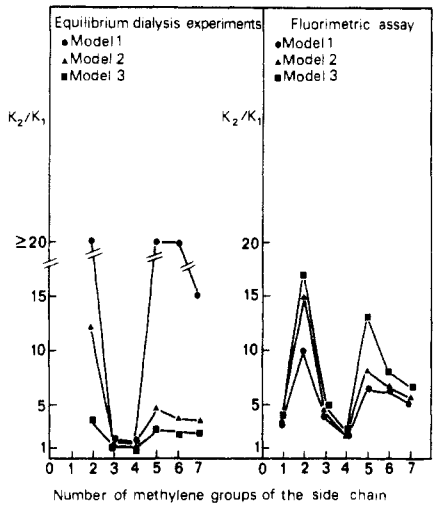

FIGURE 3: Effect of the length of the side chain of a series of 2-methoxy-6-chloro-9-[(ω-carbamoylalkyl)amino]acridine on the apparent ratio of binding affinities to preferred (K_2) and nonpreferred (K_1) sites (K_2/K_1) deduced respectively from models 1, 2, and 3, using either equilibrium dialysis or fluorometric experiments.

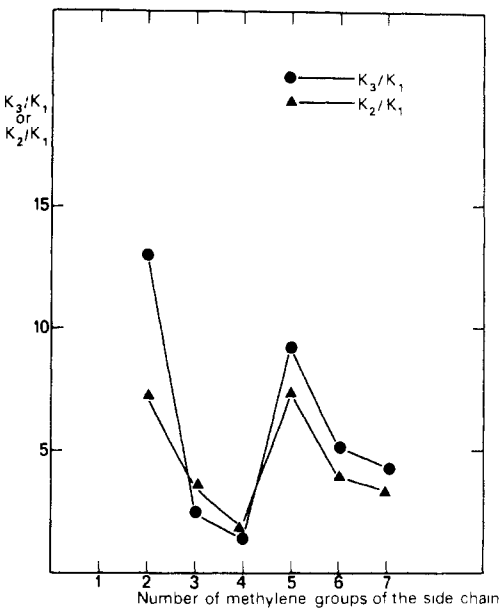

FIGURE 4: Interpretation of fluorometric and equilibrium dialysis data according to model 4. Model 4 applies if the acridine ring is specific to pyrimidine-(3′–5′)-purine base sequences. The values of K_3, K_2, and K_1 refer to the binding of the dye to CpG, TpG or CpA, and TpA sequences, respectively. The values of K_3/K_1 and K_2/K_1 computed from eq 12 and 13 are plotted for the different 2-methoxy-6-chloro-9-[(ω-carbamoylalkyl)amino]acridine compounds as a function of their chain length.

dialysis or fluorometric assay. This comparison is done as a function of the length of the chain bearing the carboxamide group. From these results it can be seen that the theoretical values of K_2/K_1 are strongly dependent on the assumed model, especially for equilibrium dialysis experiments. On the other hand, it can be noticed that the apparent value of the ratio K_2/K_1 decreases from model 1 to 3 in the case of equilibrium dialysis determinations. By contrast, this apparent ratio increases from model 1 to 3 in the case of the fluorometric assay.

From the NMR studies presented in the preceding paper (Gaugain et al., 1981), the carboxamide group borne by the side chain of these acridine derivatives is expected to confer a G specificity to these compound because this group forms hydrogen bonds with guanine at the minihelix level. Therefore if the aromatic ring can intercalate equally well in any kind of site and if there is no preferential orientation for the side chain, each site adjacent to a GC base pair will be recognized as a preferred site, in which case model 1 would apply perfectly. Nevertheless, this model does not seems to account for the experimental data. Equilibrium dialysis suggests an almost infinite preference for the GC site. Obviously the formation of two additional hydrogen bonds cannot increase the binding affinity for a GC site by so large a factor. This model does not account for experimental data. This means that each of the GC sites might not be recognized equally as well by these compounds. Restrictions on some sites could come either from the side chain or from the intercalated ring. If the side chain is free to rotate with respect to the acridine ring, the terminal carboxamide group can form hydrogen bonds with a guanine located either above or below the intercalated acridine unhindered. On the other hand, if there is a specific conformation of the side chain, the carboxamide group will be located above or below the intercalated ring and a guanine will only interact with the carboxamide if its location corresponds to the specific orientation of the side chain, in which case model 2 will apply. Experimental results could be reasonably fitted along the lines of this model. Nevertheless the NMR studies presented in the preceding paper show that the chain has no conformational preference. Therefore model 2, while being in rough agreement with experimental data, seems to have no physical meaning.

It appears therefore that the restriction on some of the sites arises from specificity related to the acridine ring. Indeed it has been shown in the preceding paper (Gaugain et al., 1981) that the acridine pentylcarbamoyl derivative exhibits a pyrimidine-(3′–5′)-purine specificity at the minihelix level.

If we assume that the pyrimidine–purine sequence is the only binding sequence for low values of r, we find then that only three types of sites are involved:

(T:A) / (A:T)	occurring with frequency $p^2/4$ and binding constant K_1
(C:G) / (A:T)	occurring with frequency $2p(1-p)/4$ and binding constant K_2
(C:G) / (G:C)	occurring with frequency $(1-p)^2/4$ and binding constant K_3

By fitting these frequency values into 4 and 5 we get

$$\alpha = \frac{K_1p_1^2 + 2K_2(p_1 - p_1^2) + K_3(1 - p_1)^2}{K_1p_2^2 + 2K_2(p_2 - p_2^2) + K_3(1 - p_2)^2} \quad (12)$$

$$\frac{I - I_{GC}}{I_{AT} - I_{GC}} = \frac{K_1p^2}{K_1p^2 + 2K_2(p - p^2) + K_3(1 - p)^2} \quad (13)$$

This pair of equations can be solved for K_3/K_1 and K_2/K_1. In Figure 4 we see the variation of K_3/K_1 and K_2/K_1 deduced for this model as a function of the length of the chain bearing the carboxamide group.

The experimental results are well accounted for in this model. Nevertheless, this cannot be taken as a proof that such a strong pyrimidine-(3′–5′)-purine sequence specificity occurs at the DNA level. However, as we will see later, those results when combined with other experiments suggest some pyrimidine-(3′–5′)-purine sequence specificity.

If the selectivity of the dyes comes from their ability to form two hydrogen bonds between guanine and the carboxamide group, we expect this selectivity to depend on the length of the side chain which bears the carboxamide group. The chain would need to contain at least five methylene groups. Furthermore this selectivity is also expected to be reduced by blocking one of the hydrogen-bond donor groups. This is obtained with derivatives with a side chain bearing a methyl

ester or a *N,N*-dimethylcarboxamide. The removal of the carboxamide group from the side chain should suppress all selectivity. Our results agree with these prediction. The results obtained with the derivative bearing a carboxamide group on a chain two carbons long are puzzling because model building studies show that, in this case, only one hydrogen bond can be formed between the side chain and the guanine base when the dye is intercalated next to a GC pair. On the other hand, compound **2**, which bears an ester group instead of a carboxamide group on the same chain, exhibits the same GC specificity. This suggests that, in the case in hand, a single hydrogen bond between the carbonyl group of the side chain and the guanine NH_2 group is enough to confer G specificity. This point will be discussed later in greater detail.

(3) Binding Parameters to Poly[d(A-T)]·Poly[d(A-T)]. Analysis of the binding of these derivatives to poly[d(A-T)]·poly[d(A-T)] (results not shown) shows that the binding parameters are almost identical for the derivatives whatever the nature of the terminal group of the side chain. On the other hand, Table I shows how compound **8**, an acridine with a side chain bearing a carboxamide group, has a lower binding affinity for poly[d(I-C)]·poly[d(I-C)] than for poly[d(G-C)]·poly[d(G-C)]. These results give further evidence in support of the involvement of the NH_2 of the guanine in hydrogen bonding with the carboxamide group of the acridine side chain.

Discussion

The purpose of this work was to try to demonstrate that the addition of a chain bearing a carboxamide group to a DNA ligand will promote base specificity. The rationale was that the carboxamide group as suggested by Seeman et al. (1976) and Hélène (1977) would be able to form two hydrogen bonds with the guanine base if the chain lies in the small groove or with the adenine base if the chain lies in the large groove. In the preceding paper the formation of complexes between acridine derivatives bearing a chain with a carboxamide group (acridine alkylamide) and RNA minihelices was demonstrated by using NMR spectroscopy. It was necessary to try to extrapolate these results at the level of the polymers. The study could have been performed either with homopolymers or with natural DNAs of various base compositions. The results seemed easier to interpret with homopolymers because of their base sequence homogeneity. Nevertheless, these polymers were well-known to adopt secondary structures quite different from those of natural DNAs (Davies & Baldwin, 1963; Arnott, 1970; Wells et al., 1970; Bram, 1971; Pohl & Jovin, 1972; Arnott & Selsing, 1974; Arnott et al., 1974; Shindo et al., 1979; Simpson & Shindo, 1980). Consequently, the interactions of ligands with such polymers could have been more dependent on their secondary structure than on their specific sequence (Capelle et al., 1979). Therefore it was necessary to perform these studies with natural DNAs. Obviously in that case the interpretation of the results is dramatically complicated by the heterogeneity of the DNA sequence since there are ten different possible intercalation sites occurring with different frequencies.

A qualitative analysis of the results clearly shows the following: (i) Acridine derivatives with an aminopentyl chain, **13**, or with an hexyl chain, **14**, elicit no base specificity. (ii) Some acridine derivatives bearing a carboxamide group in their side chain exhibit significant GC base pair specificity (compounds **5**, **8**, **9**, **10**, and **11**). The GC specificity of these dyes is almost suppressed when the two hydrogens of the carboxamide group are replaced by methyl groups but preserved when only one hydrogen is replaced by a methyl group. (iii) The GC specificity of the acridine alkylamide is strongly modulated by the length of the side chain.

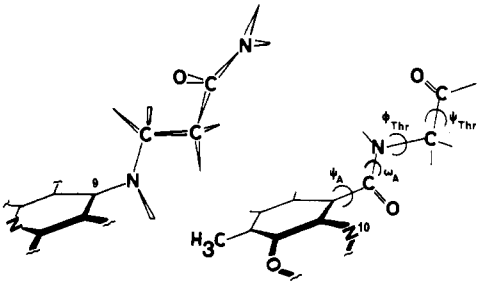

FIGURE 5: Comparison of the respective position of the carbonyl group in the acridine ethylamide (compound **5**) (left) and of the threonyl carbonyl in actinomycin D (right) relative to the corresponding intercalating ring acridine or phenoxazone. The conformation of actinomycin D is taken from Jain & Sobell (1972).

The examination of space-filling models of an intercalated complex of acridine alkylamide shows that in order to permit the interaction of the carboxamide group of the side chain through two hydrogen bonds with a G base, the chain must be at least five carbons long. In agreement with this model, we observe a sudden increase in GC specificity when the chain length increases from four to five methylene groups. Furthermore, the comparison of GC specificity between compounds **8**, **11**, **3**, and **12** suggests that two hydrogen bonds are required to confer GC specificity for this length of chain. This goes along with Seeman et al. (1976) and Hélène (1977) models.

The results obtained with the acridine ethylamide **5** are at first surprising since the space-filling model shows that only one hydrogen bond can be formed between the carbonyl group of the amide and the NH_2 of guanine.

Interesting enough, Figure 5 shows that the carbonyl group of acridine ethylamide can be positioned in an arrangement quite similar to the carbonyl of L-threonine of actinomycin D. According to Jain & Sobell (1972), it is this carbonyl which interacts with the NH_2 of guanine in DNA. The stability of this hydrogen bond is probably much greater than in the above-mentioned case because of the very limited mobility of the carbonyl group on the short chain.

Examination of the different binding models for these acridine derivatives and their physical meaning lead us to propose that the pyrimidine-(3′–5′)-purine sequence specificity of the acridine ring, like that of other intercalating compounds, occurs at both the DNA and minihelice level. The existence of such a specificity also agrees with recent theoretical calculations (Broyde & Hingerty, 1979) as well as with NMR studies and X-rays determinations (Krugh et al., 1979; Patel et al., 1979; Klug et al., 1979; Arnott et al., 1980) showing that the conformation of polynucleotides is different at pyrimidine-(3′–5′)-purine and at purine-(3′–5′)-pyrimidine sequences. Such specificity could also occur in DNA protein recognition process as already discussed by Klug et al. (1979). It has been known for some time that deoxyribonuclease 1 is specific to the pyrimidine–purine sequence [for a review see Laskowski (1971)].

The additional free energy of interaction contributed by the side chain can be computed from the ratio K_1/K_2 (from model 2). Our results suggest that if hydrogen bonds contribute to the binding specificity of these acridine compounds, two hydrogen bonds on a flexible chain or one hydrogen bond on a rigid chain contribute about 1 kcal/mol, a reasonable value in aqueous medium.

Results concerning the DNA binding of *lac* repressor or RNA polymerase indicate that these proteins bind to non-

specific DNA sites (deHaseth et al., 1977, 1978) with a binding constant 10^6 less than that for specific DNA sites. If hydrogen bonds involving guanine, adenine, asparagine, and glutamine as suggested by Seeman et al. (1976) and Hélène (1977) occur in these complexes and if the free energy of these hydrogen bonds are similar to those observed here, five to eight contacts will be sufficient to confer such high specificity.

Acknowledgments

The excellent technical assistence of J. Couprie has been very much appreciated.

Supplementary Material Available

Description of the synthesis of a series of 2-methoxy-6-chloro-9-[(ω-carbamoylalkyl)amino]acridines and corresponding methyl esters and of the various acridine derivatives referred in the text and analytical data of the various synthesized chemicals (Table I) (7 pages). Ordering information is given on any current masthead page.

References

Arndt-Jovin, D. J., Latt, S. A., Striker, G., & Jovin, T. M. (1979) *J. Histochem. Cytochem. 27*, 87–95.
Arnott, S. (1970) *Prog. Biophys. Mol. Biol. 21*, 265–319.
Arnott, S., & Selsing, E. (1974) *J. Mol. Biol. 88*, 509–521.
Arnott, S., Chandrasekaran, R., Hukins, D. W. L., Smith, P. J. C., & Watts, L. (1974) *J. Mol. Biol. 88*, 523–533.
Arnott, S., Chandrasekaran, R., Birdsall, D. L., Leslie, A. G. W., & Ratliff, R. L. (1980) *Nature (London) 283*, 743–745.
Bram, S. (1971) *Nature (London), New Biol. 232*, 174–176.
Broyde, S., & Hingerty, B. (1979) *Biopolymers 18*, 2905–2910.
Capelle, N., Barbet, J., Dessen, P., Blanquet, S., Roques, B. P., & Le Pecq, J. B. (1979) *Biochemistry 18*, 3354–3361.
Davies, D. R., & Baldwin, R. L. (1963) *J. Mol. Biol. 6*, 251–255.
deHaseth, P. L., Gross, C. A., Burgess, R. R., & Record, M. T., Jr. (1977) *Biochemistry 16*, 4777–4783.
deHaseth, P. L., Lohman, T. M., Burgess, R. R., & Record, M. T., Jr. (1978) *Biochemistry 17*, 1612–1622.
Gaugain, B., Markovits, J., Le Pecq, J. B., & Roques, B. P. (1981) *Biochemistry* (preceding paper in this issue).
Hélène, C. (1977) *FEBS Lett. 74*, 10–13.
Jain, S. C., & Sobell, H. M. (1972) *J. Mol. Biol. 68*, 1–20.
Klug, A., Jack, A., Viswamitra, M. A., Kennard, O., Shakked, Z., & Steitz, T. A. (1979) *J. Mol. Biol. 131*, 669–680.
Krugh, T. R., Hook, J. W., III, Lin, S., & Chen, F. M. (1979) in *Stereodynamics of Molecular Systems* (Sarma, R. H., Ed.) pp 423–435, Pergamon Press, New York.
Laskowski, M. S. R. (1971) *Enzymes, 3rd Ed. 4*, 289–311.
Le Pecq, J. B., Le Bret, M., Barbet, J., & Roques, B. P. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 2915–2919.
Müller, W., & Crothers, D. M. (1975) *Eur. J. Biochem. 54*, 267–277.
Müller, W., Crothers, D. M., & Waring, M. J. (1973) *Eur. J. Biochem. 39*, 223–234.
Pachmann, U., & Rigler, R. (1972) *Exp. Cell. Res. 72*, 602–608.
Paoletti, J. (1972) Thèse Doctorat ès Sciences, University of Paris.
Patel, D. J., Canuel, L. L., & Pohl, F. M. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2508–2511.
Pohl, F. M., & Jovin, T. M. (1972) *J. Mol. Biol. 67*, 375–396.
Ramstein, J., & Leng, M. (1975) *Biophys. Chem. 3*, 234–240.
Ramstein, J., Dourlent, M., & Leng, M. (1972) *Biochem. Biophys. Res. Commun. 47*, 874–882.
Revet, B., Schmir, M., & Vinograd, J. (1971) *Nature (London), New Biol. 229*, 10–23.
Saucier, J. M., Festy, B., & Le Pecq, J. B. (1971) *Biochimie 53*, 973–980.
Seeman, N. C., Rosenberg, J. M., & Rich, A. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 804–808.
Shindo, H., Simpson, R. T., & Cohen, J. (1979) *J. Biol. Chem. 254*, 8125–8128.
Simpson, R. T., & Shindo, H. (1980) *Nucleic Acids Res. 8*, 2093–2103.
Weisblum, B., & deHaseth, P. L. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 629–632.
Wells, R. D., Larson, J. E., Grand, R. C., Shortle, B. E., & Cantor, C. R. (1970) *J. Mol. Biol. 54*, 465–497.