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DNA Tris-Intercalation: First Acridine Trimer with DNA Affinity in the Range of DNA Regulatory Proteins. Kinetic Studies†

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ABSTRACT: A trimer made up of three acridine chromophores linked by a positively charged poly(aminoalkyl) chain was synthesized as a potential tris-intercalating agent. The length of the linking chain was selected to allow intercalation of each chromophore according to the excluded site model. ^{1}H NMR studies have shown that, at 5 mM sodium, pH 5, the acridine trimer occurred under a folded conformation stabilized by stacking interactions between the three aromatic rings. DNA tris-intercalation of the dye at a low dye/base pair ratio was shown by measurements of both the unwinding of PM2 DNA and the lengthening of sonicated rodlike DNA. The trimer exhibits a high DNA affinity for poly[d(A-T)] ($K_{app} = 8 \times 10^8$ M^{-1}, 1 M sodium) as shown by competition experiments with ethidium dimer. Kinetic studies of both the association with poly[d(A-T)] and the exchange between poly[d(A-T)] and sonicated calf thymus DNA have been performed as a function of the ionic strength. In 0.3 M sodium the on-rate constant ($k_1 = 2.6 \times 10^7$ M^{-1} s^{-1}) is similar to that reported for other monoacridines or bis(acridines), whereas the off-rate constant is much smaller ($k_{-1} = 1.2 \times 10^{-4}$ s^{-1}), leading to an equilibrium binding constant as large as $K_{app} = 2.2 \times 10^{11}$ M^{-1}. A plot of log (k_1/k_{-1}) as a function of log [Na$^+$] yielded a straight line whose slope shows that 5.7 ion pairs (out of 7 potential) are formed upon the interaction with DNA. From this linear relationship a K_{app} value of 10^{14} M^{-1} in 0.1 M sodium can be estimated. Such a value reaches and even goes beyond that of some DNA regulatory proteins. This acridine trimer appears to be the first synthetic ligand with such a high DNA affinity.

Gene expression mainly depends upon interactions between nucleic acids and regulatory proteins. Such interactions are characterized by both high affinity and high specificity (Riggs et al., 1970; Lin & Riggs, 1972). Therefore, compounds that aim to interfere with genetic expression should ideally present these two properties.

On one hand, it has been shown that base specificity can be achieved by means of hydrogen bonding between nucleic acid bases and donor or acceptor groups borne by the ligand (Seeman et al., 1976; Hélène, 1977; Ohlendorf et al., 1982).

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This is indeed the case for actinomycin D (Jain & Sobell, 1972) or for 9-(carboxamidoalkyl)acridine derivatives (Gaugain et al., 1981; Markovits et al., 1981a). However, a consistent gain in both specificity and affinity would require the formation of at least five to eight hydrogen bonds, between the intercalating agent and an appropriate DNA sequence. The requirements for a perfect positioning of the hydrogen-bonding groups render difficult the design of such a molecule.

On the other hand, it has been demonstrated that dimerization of intercalating compounds can lead to molecules with enhanced DNA affinity (Le Pecq eq al., 1975; Gaugain et al., 1978). Among these molecules several antitumoral agents have been discovered such as Ditercalinium (NSC 335153), in the series of 7*H*-pyridocarbazole, which is now introduced in phase I clinical trial (Roques et al., 1979; Pelaprat et al., 1981; Esnault et al., 1984). In an effort to obtain molecules with still larger affinities, acridine trimers have been recently synthesized (Atwell et al., 1983; Hansen & Buchardt, 1983; Hansen et al., 1983; Gaugain et al., 1984). DNA binding data have been reported for only two acridine trimers (Atwell et al., 1983; Gaugain et al., 1984). It has been shown that both trimers bearing carboxamidoalkyl-linking chains tris-intercalate into DNA though differing from the spacing between two consecutive chromophores: 7 (Atwell et al., 1983) and 17 Å (Gaugain et al., 1984). Their DNA affinities are larger than 10^6 (Atwell et al., 1983) and 2×10^8 M^{-1} (Gaugain et al., 1984). It should be noted that the compound described by Gaugain et al. (1984) has lost the guanine specificity elicited by the corresponding monomer (Markovits et al., 1981a; Gaugain et al., 1984). Moreover, the binding constant undergoes only a 10-fold increase on going from the dimer to the trimer.

Such results underline the importance of the nature of the linking chain since acridine dimers with positively charged polyamine chains possess binding affinities several orders of magnitude higher. This is likely related to additional ionic interactions between the linking chain and the sugar phosphate backbone of DNA and enhanced flexibility due to the absence of amide groups (Le Pecq et al., 1975; Capelle et al., 1979; Markovits et al., 1981b).

We were therefore led to synthesize an acridine trimer, AcTri2 (Figure 1), bearing an aminoalkyl chain obtained through hydrogenation of the carboxamidoalkyl chain of the foregoing trimer AcTri1, described by Gaugain et al. (1984).

This work presents a brief description of the synthesis and NMR-derived conformational properties of AcTri2 and a detailed investigation of its DNA binding behavior which is compared to that of both AcTri1 and the closest amino alkyl acridine dimer, AcDi, whose structure is shown in Figure 1 (Barbet et al., 1975). Classical hydrodynamic experiments strongly suggest that AcTri2 tris-intercalates into DNA at a low dye/base pair ratio. Besides, competition and association–dissociation experiments show that a positively charged polyamine chain considerably increases the DNA affinity of the dye, via a very long dissociation rate from DNA.

Experimental Procedures

Materials

Synthesis. The synthesis of AcTri2, summarized in Figure 2, is reported in the supplementary material (see paragraph at end of paper regarding supplementary material).

Nucleic Acids. DNAs from calf thymus, PM2 (Boehringer), *Micrococcus luteus*, and *Clostridium perfringens* (Sigma) were purified by three phenol extractions. Poly[d(A-T)] and poly[d(G-C)] (Boehringer) were used without purification.

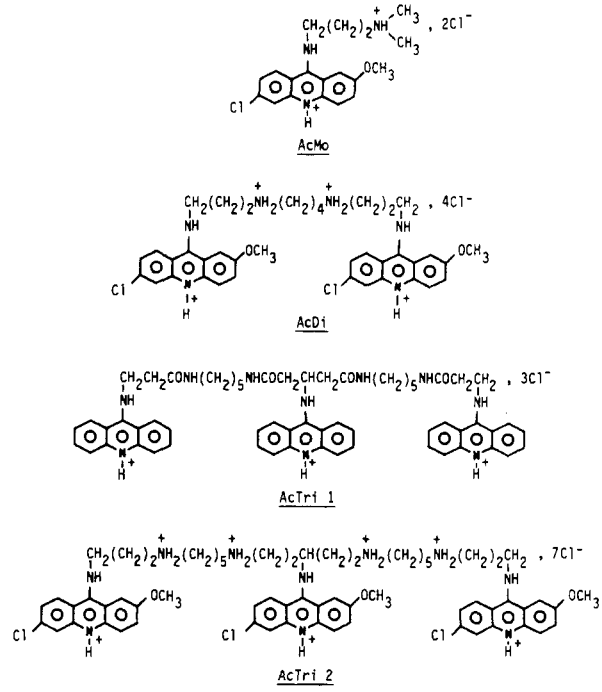

FIGURE 1: Chemical structures of the acridine monomer (AcMo), dimer (AcDi), and trimers (AcTri1 and AcTri2).

Sonicated calf thymus DNA was prepared as described by Saucier et al. (1971).

Molar DNA nucleotide concentrations were determined by using the following extinction coefficients at 260 nm: calf thymus, 6400 M^{-1} cm^{-1}; *M. luteus*, 6900 M^{-1} cm^{-1} (Müller & Crothers 1975); *C. perfringens*, 6230 M^{-1} cm^{-1}; poly[d(A-T)], 6800 M^{-1} cm^{-1}; poly[d(G-C)], 8400 M^{-1} cm^{-1} at 254 nm (Wells et al., 1970). AcTri2 concentration was measured by using the maximum extinction coefficients at pH 5.0 of ϵ_{424} = 16 500 M^{-1} cm^{-1} and ϵ_{275} = 90 000 M^{-1} cm^{-1}.

Methods

All experiments were performed at 25 °C in a sodium acetate buffer, pH 5.0.

NMR Studies. ^1H NMR spectra were recorded in 5 mM sodium deuterioacetate buffer (99.97% ^2H_2O) at 270 MHz with a Brucker WH 270 spectrometer operating in the Fourier transform mode. The chemical shifts are referenced to an internal standard of 4,4-dimethyl-4-silapentane-1-sulfonate (DSS). Temperature was monitored by a B-ST 100/700 controller unit. The residual water resonance was reduced by standard homonuclear gated decoupling.

Viscosimetry. Viscosimetric measurements were performed in a semimicrodilution capillary viscosimeter (Cannon Instrument Co., State College, LA).

The unwinding of DNA helix was measured by using covalently closed circular PM2 DNA (Revet et al., 1971).

The length increase of short calf thymus DNA segments was measured by the increase of intrinsic viscosity η of the DNA in the presence of increasing concentrations of AcTri2 as reported by Saucier et al. (1971).

Fluorescence Measurements. Measurements were made in an SLM 800 spectrofluorometer (Urbana, IL) equipped with a thermostated cell holder. This instrument was associated with an 9315 Ortec photon counter interfaced to a Minc digital computer. When necessary the fluorescence signal was improved by the use of quartz cuvettes with two silver-stained faces (Hellma).

Stopped-Flow Measurements. A Durrum Gibson D110 stopped-flow instrument equipped with fluorescence detection

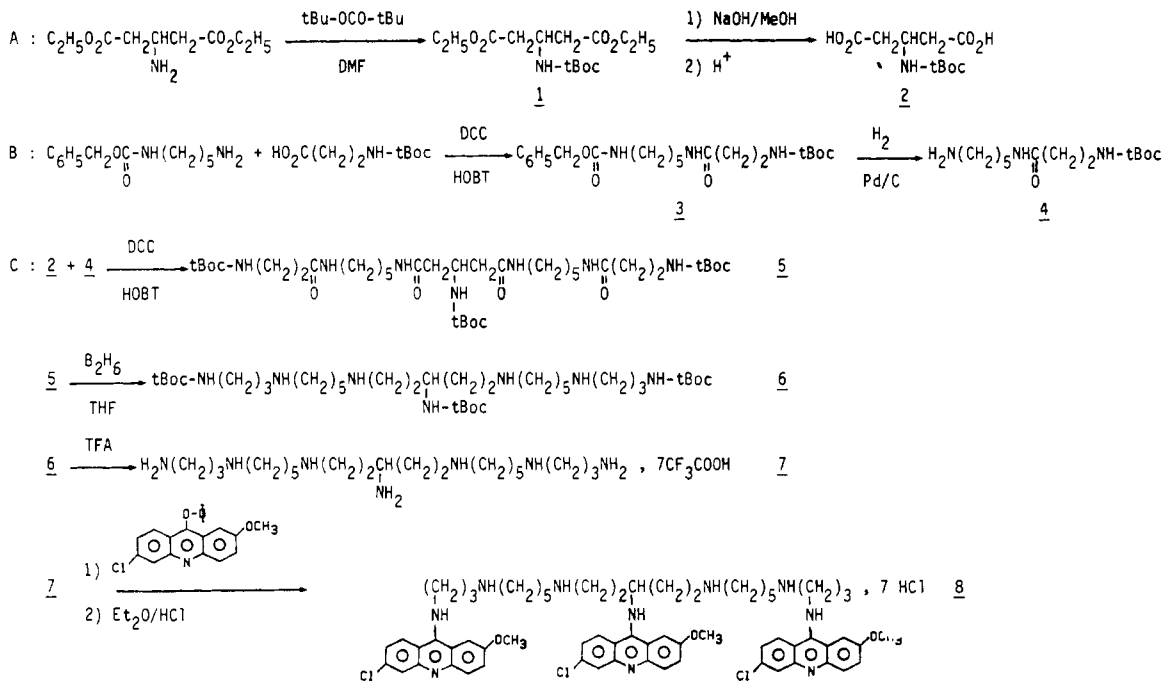

FIGURE 2: Scheme for the synthesis of AcTri2.

interfaced to a Minc digital computer was used. The mixing dead time of the instrument was less than 2.5 ms (Markovits et al., 1983). The excitation wavelength was set at 450 nm. Fluorescence emission (500 nm) was observed through a yellow filter. The apparatus was thermostated at 25 °C.

Computations. Data stored in the Minc computer were analyzed by nonlinear regression by using the Marquardt algorithm for single- and double-exponential processes (Marquardt, 1963). To decide whether a single- or a double-exponential process statistically improved the fitting of the data, an *F* test was performed according to Bevington (1969).

Association Kinetics. The on-rate constants at various ionic strengths (0.1–2 M) were directly measured in the stopped-flow apparatus, as already described (Capelle et al., 1979).

Exchange Kinetics. The dissociation rates from poly[d(A-T)] were determined at various ionic strengths (0.3–2 M) from the exchange kinetics of the dye between poly[d(A-T)] and sonicated calf thymus DNA (42% G·C) (Capelle et al., 1979).

AcTri2 (3×10^{-8} M) was first preequilibrated with poly[d(A-T)] (3×10^{-6} M nucleotide) for at least 16 h in pH 5 sodium acetate buffer of appropriate ionic strength. Then equilibrium was perturbed by the addition of an excess (40-fold) of sonicated calf thymus DNA in the same buffer.

At low ionic strength (0.3–1 M) the fluorescence variations were followed in the SLM 800 spectrofluorometer (λ_{exc} 450 nm; λ_{em} 500 nm). Two hundred points were stored by the computer, the first hundred being collected at a higher frequency than the last.

Since at high ionic strength (1–2 M) short relaxation rates were observed, the exchange kinetics were monitored in the stopped-flow instrument following the previously reported procedure (Markovits et al., 1983). To improve the signal molar concentration of the reactants was 3 times that used with the SLM 800 apparatus.

In both cases data were stored and analyzed under the computer control as described above. Similar results were found in 1 M Na^+ with both instruments.

Competition Experiments. The equilibrium binding constant in 1 M sodium acetate buffer, pH 5.0, was determined by competition experiments with ethidium dimer as previously reported (Gaugain et al., 1978).

Results and Discussion

Synthesis. Acridine trimer **8** (AcTri2) was synthesized according to the scheme in Figure 2. A symmetrical aminoalkyl-linking chain (**7**) bearing three primary amino groups was prepared to be condensed with 3 equiv of acridine chromophore. The chain was chosen long enough to obtain a favorable spacing distance (17 Å) between two consecutive acridine moieties allowing tris-intercalation of the dye into DNA.

Chain **7** was built by condensation of a central link (**2**) with two lateral chains (**4**).

The synthesis of the central link starts with diethyl 3-aminoglutarate hydrochloride (Rosowsky et al., 1976) whose primary amino group is protected with di-*tert*-butyldicarbonate in dimethylformamide in the presence of triethylamine to yield diethyl 3-[(*tert*-butyloxycarbonyl)amino]glutarate (**1**). The latter is saponified and upon hydrolysis yields 3-[(*tert*-butyloxycarbonyl)amino]glutaric acid (**2**).

The synthesis of the lateral chains (**4**) starts with 5-[(benzyloxycarbonyl)amino]pentylamine, prepared according to Clarke et al. (1959), which is coupled with 3-[(*tert*-butyloxycarbonyl)amino]propionic acid by means of dicyclohexylcarbodiimide and hydroxybenzotriazole, as in classical peptide synthesis, yielding 9-[(benzyloxycarbonyl)amino]-1-[(*tert*-butyloxycarbonyl)amino]-3-oxo-4-azanonane (**3**). Catalytic hydrogenation of **3** yielded 9-[(*tert*-butyloxycarbonyl)amino]-7-oxo-6-azanonylamine (**4**).

Linking chain **7** was then prepared in three steps. The first one corresponds to a peptidic coupling between central link **2** and 2 equiv of lateral chain **4** by means of dicyclohexylcarbodiimide and hydroxybenzotriazole. The amide functions of condensation product **5** [1,13,25-tris[(*tert*-butyloxycarbonyl)amino]-3,11,15,23-tetraoxo-4,10,16,22-tetraazapentacosane] were reduced with diborane in tetrahydrofuran to yield 1,13,25-tris[(*tert*-butyloxycarbonyl)amino]-4,10,16,22-tetraazapentacosane (**6**). The primary amino groups were then deprotected with trifluoroacetic acid to give linking chain **7**.

The resulting salt was coupled to 3 equiv of 6-chloro-2-

methoxy-9-phenoxyacridine following the general procedure to obtain substituted 9-aminoacridine (Dupré & Robinson, 1945). The trimer was purified by column chromatography and finally converted with ether–hydrochloric acid to the more stable and water-soluble heptahydrochloride trimer AcTri2 (**8**).

Acridine Trimer: pK_a and Conformation. The strength of DNA interaction of oligomeric intercalators is on the dependence of both the ionic and conformational states of the intercalating agent (Delbarre et al., 1981). Therefore, in a first step we have evaluated the pK_a of AcTri2 by means of visible spectroscopy in 0.1 M NaCl to determine the optimal pH value for DNA interactions.

The spectrum of AcTri2 displays considerable hypochromism in the bands at 275, 424, and 444 nm when the pH is raised. A small transition appears between pH 2.4 and pH 2.8 (17% of the total hypochromism at 424 nm, figure S1 in the supplementary material) followed by a large sharp transition between pH 5.2 and pH 6.8. At upper pH values AcTri2 precipitates, a feature probably due to the formation of insoluble aggregates following the complete deprotonation of the acridine rings (Irvin et al., 1950).

Nevertheless, according to Irvin et al. (1950) a pK_a value of about 6.2 can be estimated from the pH titration curve for the intracyclic nitrogen ring. This value is lower than that of the monomer AcMo ($pK_a \simeq 8.2$) bearing an aminoalkyl chain in position 9, but close to that of the acridine dimer, AcDi ($pK_a \simeq 6.5$) (Le Pecq et al., 1975).

In a second step the conformational behavior of AcTri2 was studied in aqueous solution by ^1H NMR spectroscopy. It is now well established that planar aromatic rings such as 9-amino-6-chloro-2-methoxyacridine strongly self-associate in water by stacking interactions between the aromatic moieties (Barbet et al., 1976; Gaugain et al., 1981). In aqueous medium at low concentration ($<10^{-3}$ M) acridine dimers exist under equilibrium between unfolded and folded conformations with the two rings stacked on each other (Barbet et al., 1976).

The conformational behavior of the acridine trimer (33 μM) was studied at 27 °C in deuterioacetate buffer, pH 5.0, and compared to that of AcMo (100 μM) and AcDi (50 μM). The different concentrations used correspond to identical concentrations in acridine rings. The spectra (Figure 3) of AcMo and AcDi were assigned according to Barbet et al. (1976). In the trimer spectrum the well-resolved doublet of two protons area, observed at 8.03 ppm, was tentatively assigned to H_8 protons of the two external acridine rings (see arrows on Figure 3) owing to the symmetry of the molecule and by comparison with AcMo and AcDi spectra. The resonances of the 16 remaining protons overlap in a cluster between 7.2 and 7.6 ppm precluding their complete assignment. Nevertheless, thanks to homodecoupling experiments, the H_7 signals corresponding to the external rings were found at 7.26 ppm on the upfield border of the cluster. No attempt at an extensive identification of the chain protons was made in the buffer.

Aromatic resonances are high field shifted on going from AcMo to AcTri2 (Figure 3). In the 100 μM concentration range, AcMo does not self-associate in water because of the low value of its stacking constant [$K_s < 100$ M^{-1} at 27 °C (Barbet et al., 1976)]. Similarly, no intermolecular association is assumed to occur with AcDi and AcTri2 since their spectra were left unchanged upon a concentration increase.

Barbet et al. (1976) have shown that the AcDi molecule is partly folded at 27 °C, which accounts for the high-field shifts of the aromatic resonances, but almost completely folded around 0 °C.

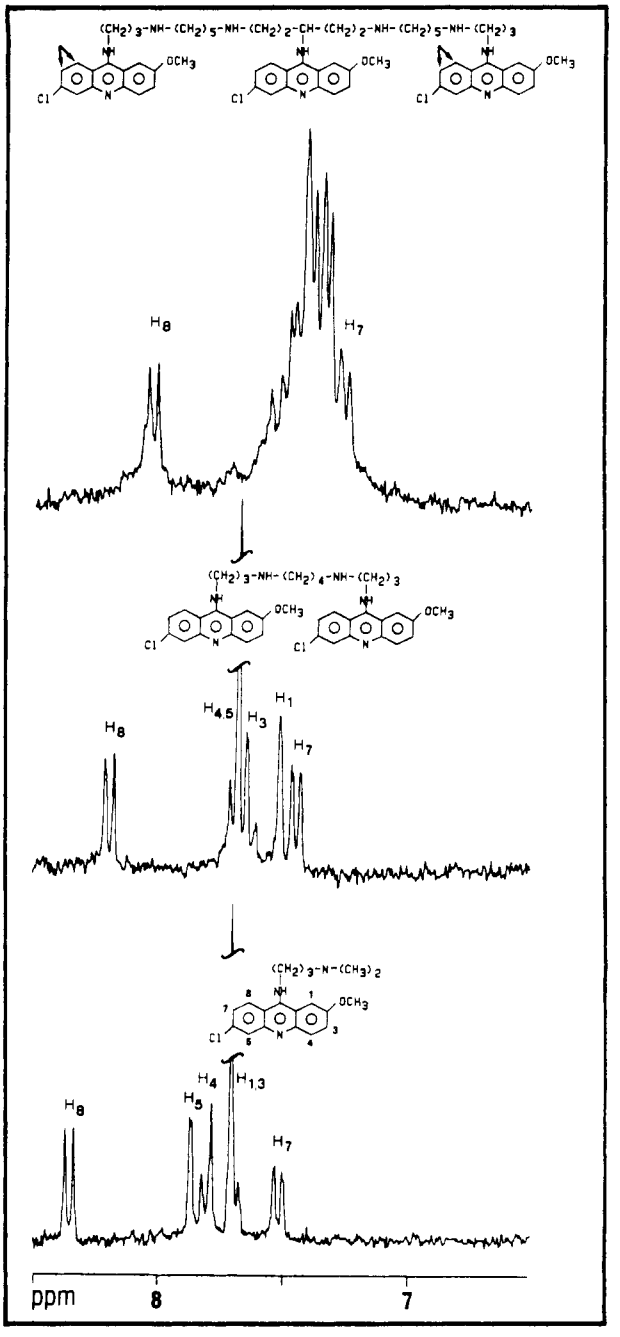

FIGURE 3: ^1H NMR (270 MHz) spectra of AcTri2 (top), AcDi (middle), and AcMo (bottom). The arrows refer to protons noted H_8 and H_7 in AcTri2 spectrum that correspond to acridine rings situated at the outside of the self-stacked trimer. The same concentration (10^{-4} M, acridine ring) was used for each experiment. All sepctra were recorded in 5 mM deuterioacetate buffer, pH 5.0 at 27 °C.

Because AcTri2 spectrum at 27 °C looks like AcDi spectrum at 4 °C (not shown here), it is then assumed that the trimer adopts a folded conformation in water.

Though a precise determination of AcTri2 geometry is not possible, it is noteworthy that (i) H_7, H_8, and OCH_3 of the external rings have chemical shifts similar to those of fully folded AcDi (upfield shifts relative to AcMo at low concentration of 0.15, 0.32, and 0.08 ppm, respectively), (ii) the central H_8 resonance is additionally upfield shifted by at least 0.8 ppm, and (iii) the H_7 and OCH_3 signals of the central ring are slightly deshielded as compared to the corresponding protons in the external acridines.

Taken together, these results strongly suggest that the

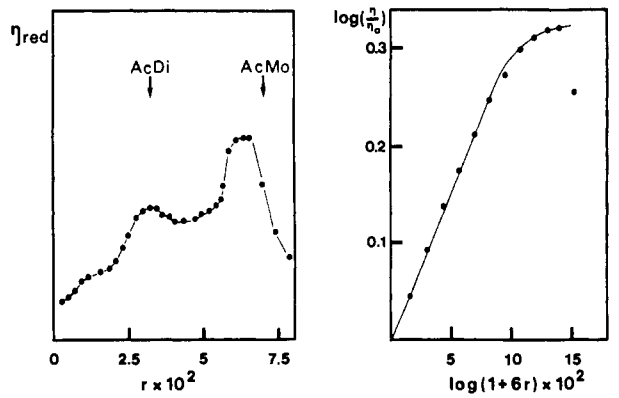

FIGURE 4: (Left) Determination of the unwinding angle of PM2 DNA helix caused by AcTri2. The reduced viscosity η_{red} is measured as a function of r, number of dye molecules bound by the nucleotide. The arrows correspond to the maximum in reduced viscosity when AcDi or AcMo is bound to PM2 DNA. (Right) Determination of the length increase of sonicated DNA caused by AcTri2. Intrinsic viscosities of DNA are measured in the absence of drug (η_0) and in the presence of increasing concentration of drug (η). r is the number of dye molecules bound by nucleotide. Both experiments were performed in 0.2 M sodium acetate buffer, pH 5.0.

central acridine moiety is embedded in the folded molecule with the external rings stacked on each side, rather than exposed to the solvent with the external rings stacked on each other. The relative deshielding of the central acridine H_7 and OCH3 protons could indicate that, in the trimer, the long axis of the central acridine ring is not parallel to that of the external rings, presumably for steric restrictions.

Three ring–ring contacts are expected to strongly stabilize the folded conformation in low ionic strength. Indeed, we did not observe any modification of AcTri2 spectrum up to 60 °C, temperature above which the trimer underwent degradation.

DNA Binding. (*a*) *Intercalation Ability.* The binding mode of AcTri2 to DNA was studied by viscosimetric measurements of both the unwinding angle of PM2 DNA helix and the length increase of sonicated DNA upon AcTri2 binding in 0.2 M sodium acetate buffer, pH 5.0. The results of the measurements are presented in Figure 4. They both bring evidence for AcTri2 tris-intercalation.

(*i*) *DNA Lengthening.* According to Saucier et al. (1971) intercalation of an aromatic dye between DNA base pairs induces a lengthening of DNA which is related to r, the number of bound dye per nucleotide, through the relation

$$\log (\eta/\eta_0) = m \log (1 + 2r)$$

where η and η_0 are the intrinsic viscosities of the solution in the presence and in the absence of drug. Extension of this relation to an N-mer able to intercalate n of its N chromophores yields

$$\log(\eta/\eta_0) = m \log (1 + 2nr)$$

The slope m is expected to be in the range 2–3 for classical intercalating compounds (for ethidium, $N = 1$, $n = 1$, and $m = 2.3$; for AcMo, $N = 1$, $n = 1$, and $m = 2.1$; for AcDi, $N = 2$, $n = 2$, and $m = 2.4$). However, this slope can be higher in the case of large ligands able to induce rigidification of the DNA helix, like actinomycin D ($N = 1$, $n = 1$, and $m = 3.7$) (Müller & Crothers, 1968).

With regard to AcTri2 such a representation yielded a straight line up to $r = 4 \times 10^{-2}$ with a slope $m = 3$ and $n = 3$ (Figure 4, right), bringing evidence for tris-intercalation of this trimer. It can be observed that the m values increase on going from AcMo to AcTri2, which could indicate a progressive rigidification of the DNA helix induced by the growing size of the overall linking chain.

All viscosimetric measurements have been performed in conditions (0.2 M sodium) in which the life time of the complex is larger than 24 h (see kinetic studies). Therefore, no redistribution of the dye along the polymer can take place during the experiment. When r is larger than 4×10^{-2} (one dye bound per 25 nucleotides, i.e., 6 base pairs occupied out of 12), very few sites remain available for tris-intercalation (less than 15%). This compels the trimer to bis- or mono-intercalate. Such a feature is evidenced by the sudden flattening of the lengthening curve when r goes beyond 4×10^{-2}.

Attempts to perform the experiments in higher ionic strength to weaken the interaction with DNA failed because the high DNA concentration (3×10^{-4} M) required in these experiments induced precipitation in the capillary.

(*ii*) *Unwinding of PM2 DNA.* When the dye to nucleotide ratio (r) is increased, the viscosity of PM2 DNA exhibits three transitions (Figure 4, left) corresponding to unwinding angles 66 ± 10°, 41 ± 3°, and 20 ± 1°. These values were computed on the basis of an unwinding angle of 26° and an equivalence point $r_e = 0.047$ measured for ethidium, in the same experimental conditions.

An unwinding angle of 66° is consistent with tris-intercalation of AcTri2. However, when r becomes larger than 2×10^{-2}, AcTri2 no longer behaves like a tris-intercalator but presents characteristic features of bis- and even mono-intercalators, i.e., unwinding angles of 41° and 20° that should be compared to 38° and 17° for AcDi and AcMo. Since these measurements were also performed in 0.2 M sodium salt, thus out of thermodynamic equilibrium, the explanations set forth for DNA lengthening hold again: as r increases, the remaining free sites become statistically too small to accommodate a ligand covering six base pairs and the binding of the dye progressively slides from tris- to mono-intercalation. The presence of two well-defined maxima at 41° and 20° in the viscosity pattern of PM2 DNA suggests that each intercalation mode occurs at the expense of the foregoing. However, the displacement toward bis- and mono-intercalation is observed for an r value smaller than with sonicated rodlike DNA ($r \simeq 2 \times 10^{-2}$ vs. 4×10^{-2}), suggesting that tris-intercalation of such a large ligand is unfavored by the highly constrained state of PM2 DNA (one superhelical turn per 100 base pairs).

Nevertheless, both experiments show that at a low dye to nucleotide ratio ($r < 2 \times 10^{-2}$–4×10^{-2}) AcTri2 tris-intercalates into DNA.

(*b*) *DNA Affinity.* All DNA binding studies have been performed at pH 5.0, below the pK_a of AcTri2 to maximize ionic interactions. Moreover, this allows straightforward comparison with studies yet performed with AcMo and AcDi at pH 5.0 (Le Pecq et al., 1975; Capelle et al., 1979).

In a first step the size of the DNA binding site of AcTri2 has been determined by measuring the fluorescence of the dye bound to DNAs of various A-T content [poly[d(A-T)], *C. perfringens* (70% A-T), calf thymus (58% A-T), *M. luteus* (28% A-T), and poly[d(G-C)]], at a very low dye to nucleotide ratio ($r = 2.5 \times 10^{-3}$) where only tris-intercalation occurs. A log–log plot of the ratio of the fluorescence of DNA-bound AcTri2, $(I - I_{GC})/(I_{AT} - I_{GC})$, where subscripts GC and AT refer to poly[d(G-C)] and poly[d(A-T)], vs. the A-T fraction of the polymer yielded a straight line (not shown) with a slope equal to 5.7 ± 0.2 ($r^2 = 0.999$). This shows that AcTri2 does not elicit any base-pair specificity. Furthermore, it has been shown that such plots for acridine monomers, covering two base pairs, and acridine dimers, covering four base pairs, indeed gave straight lines with slopes respectively equal to 2 and 4

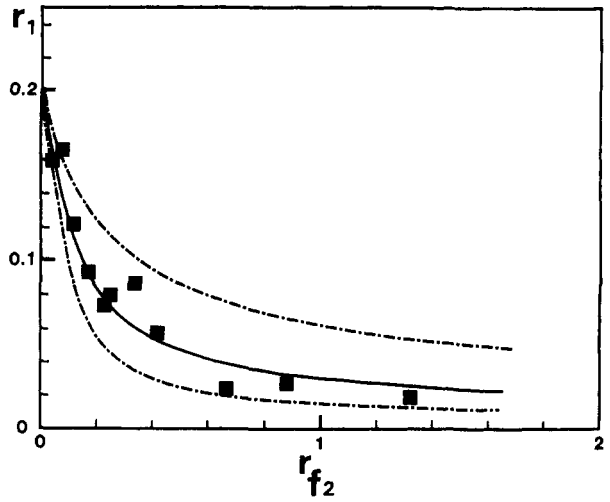

FIGURE 5: Displacement of ethidium dimer bound to poly[d(A-T)] by competing AcTri2 in 1.0 M sodium acetate buffer, pH 5.0. The concentration of bound ethidium dimer per base pair (r_1) is deduced from fluorescence measurements; r_{f2} is the ratio of the total molar concentration of AcTri2 to the molar concentration of DNA base pair. The full squares represent experimental data. The solid and dashed lines represent the theoretical curves computed according to a binding site size of six base pairs for AcTri2 and binding constants K_{app} = 8×10^8 M^{-1} (solid line), $K_{app} = 4 \times 10^8$ M^{-1} (upper dashed line), and $K_{app} = 2 \times 10^9$ M^{-1} (lower dashed line).

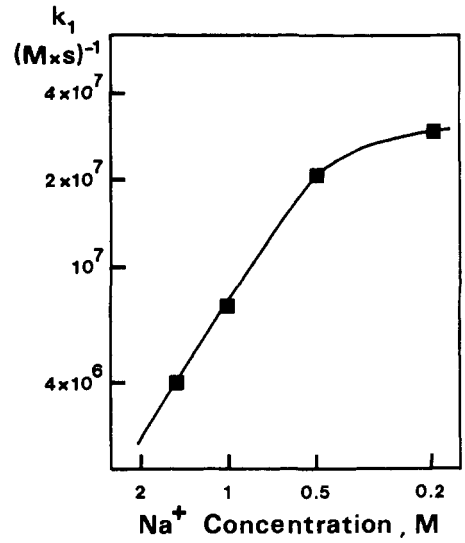

FIGURE 6: Effect of the ionic strength on the association rate k_1 of AcTri2 to poly[d(A-T)] in sodium acetate buffer, pH 5.0.

(Le Pecq et al., 1975). This is related to the fluorescence quenching of the DNA-bound dye when a single G·C base pair is present in the binding site. Therefore, a slope of 5.7 means that the binding site of AcTri2 is constituted of six base pairs.

The apparent binding constant for poly[d(A-T)] was determined by competition with ethidium dimer (Gaugain et al., 1978) in 1.0 M NaCl–acetate buffer, pH 5.0. In Figure 5 the experimental points are compared to the theoretical displacement curves calculated from McGhee and Von Hippel equations (1974) as already described (Gaugain & al., 1978). Assuming that the number of covered base pairs is equal to six, according to the fluorescence quenching data reported above, a fairly good agreement between calculated isotherm and experimental data is obtained with a K_{app} value of about 8×10^8 M^{-1}.

Furthermore, the poly[d(A-T)] binding affinity has been deduced from association–dissociation kinetics performed as a function of the ionic strength to improve the result. Indeed a K_{app} value equal to 8×10^8 M^{-1} is very near the upper limit of precision of the competition method, thus precluding any determination of the DNA binding constant at ionic strength lower than 1 M since interactions are still stronger. All these kinetic studies have been performed at low dye to nucleotide ratio so that AcTri2 tris-intercalates into DNA.

(*c*) *Association Kinetics.* Similar to aminoacridines and bis(acridines), AcTri2 binds to poly[d(A-T)] with a large fluorescence enhancement, allowing therefore to study association kinetics by use of a stopped-flow instrument equipped with fluorescence detection.

In experimental conditions of concentration for which the molar concentration of poly[d(A-T)], P_0, is large in comparison to that of AcTri2, all association kinetics fit a single exponential. The observed rate constants, k_{obsd}, vary linearly with the polymer concentration, according to the relation $k_{obsd} = k_1P_0 + k_{-1}$ which holds in the pseudo-first-order approximation, k_1 and k_{-1} being respectively the association and dissociation rate constants. As expected for a ligand eliciting a high DNA binding affinity the extrapolated values of the observed rate constants to zero DNA concentration are close to zero, precluding the determination of dissociation rates k_{-1}.

The on-rate constants, k_1, are very similar to those reported for AcTri1 as well as for acridine dimers or monomers ($k_1 = 2.9 \times 10^7$ M^{-1} s^{-1}) in the same ionic conditions (0.2 M salt) (Gaugain et al., 1984; Capelle et al., 1979). Moreover, the on-rate constant of AcTri2 also decreases as the ionic strength is increased (Figure 6).

All association rates are similar whether the dye is mono-, di-, or trimeric. Such a feature could be relevant, as suggested by Capelle et al. (1979), to the binding of the dye to preexisting kinks or loops in DNA, the association being a diffusion-controlled process weighted by the probability of opening the DNA helix. However, recent experiments (Guillerez, 1984) suggest that this unique range of on-rate constants could be related to desolvatation of the dye which necessarily occurs upon intercalation. The binding of a dye (D) to DNA (P) should be schematized as follows:

$$D_s + P_s \underset{k_{-1}'}{\overset{k_1'}{\rightleftharpoons}} D_s//P_s \underset{k_{-2}'}{\overset{k_2'}{\rightleftharpoons}} (DP)_s$$

where subscript s refers to a solvated state.

Two relaxation rates are expected:

$$\zeta_1^{-1} = k_1'(D_s + P_s) + k_{-1}' + k_2' + k_{-2}'$$

$$\zeta_2^{-1} = [k_1'k_2'(D_s + P_s)]/[k_{-1}' + k_1'(D_s + P_s)] + k_{-2}'$$

If the collision between D_s and P_s is a diffusion-controlled process, then k_1' is in the range 10^9 M^{-1} s^{-1}. According to Guillerez (1984) the complex $D_s//P_s$ is only the result of the contact between two solvatation spheres, and its stability is in the range 1 M^{-1}, leading to a dissociation constant $k_{-1}' \simeq 10^9$ s^{-1}.

The stability of the complex $(DP)_s$ is larger than 10^6 M^{-1}; therefore, $k_2' \gg k_{-2}'$. Guillerez (1984) has shown that desolvatation of aromatic dyes such as nucleic bases occurs at a rate of $k_2 \simeq 10^7$ s^{-1}. Both relaxation rates simplify to

$$\zeta_1^{-1} \simeq k_{-1}' \simeq 10^9 \text{ s}^{-1}$$

since $(D_s + P_s) \simeq (P_s) \simeq 10^{-6}$–$10^{-5}$ M. This relaxation rate is too fast to be detected.

$$\zeta_2^{-1} \simeq (k_1'/k_{-1}')k_2'(D_s + P_s) + k_{-2}'$$

$$\zeta_2^{-1} \simeq k_2'(P_s) + k_{-2}'$$

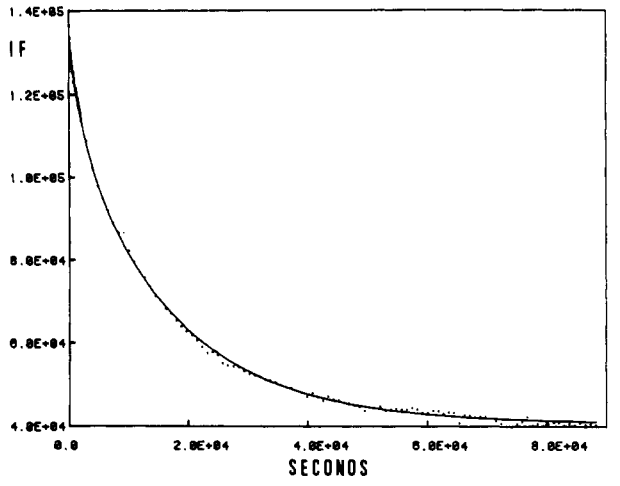

FIGURE 7: Example of a measurement of the exchange reaction between the AcTri2–poly[d(A-T)] complex and sonicated calf thymus DNA performed in 0.3 M sodium acetate buffer, pH 5.0. The concentrations were 3×10^{-8} (AcTri2), 3×10^{-6} [poly[d(A-T)], nucleotide], and 1.2×10^{-4} M (calf thymus DNA, nucleotide). The fluorescence variation, IF (arbitrary units), is plotted vs. time (seconds).

Table I: Ionic Strength Dependence of AcTri2 Binding Parameters

$[Na^+]$ (M)	k_1 (s^{-1} M^{-1})[a]	k_{-1} (s^{-1})[b]	K_{app} (M^{-1})[c]
2	2.5×10^6	8.3×10^{-2}	3×10^7
1.5	4.0×10^6	2.8×10^{-2}	1.4×10^8
1.0	7.0×10^6	7.1×10^{-3}	1×10^9
0.8	1.0×10^7	4.0×10^{-3}	2.5×10^9
0.5	2.1×10^7	5.8×10^{-4}	3.6×10^{10}
0.3	2.6×10^7	1.2×10^{-4}	2.2×10^{11}
0.2	2.9×10^7	*d*	

[a] k_1 is the rate of binding of AcTri2 to poly[d(A-T)]. [b] k_{-1} is the exchange rate of AcTri2 from poly[d(A-T)] to sonicated calf thymus DNA. All measurements were performed in sodium acetate buffer, pH 5. [c] Apparent binding constants evaluated from the kinetic measurements according to $K_{app} = k_1/k_{-1}$. [d] k_{-1} values were too small to be measured in these conditions.

since $k_1'/k_{-1}' \simeq 1$ and $(P_s) \gg (D_s)$. ζ_2^{-1} is the observed rate constant; hence, the measured association rate, k_1, is in the range of k_2' (i.e., 10^7 M^{-1} s^{-1}). Therefore, according to Guillerez (1984) the desolvatation of an aromatic dye could indeed be the rate-limiting step in the DNA binding process.

(*d*) *Exchange Kinetics.* Owing to the sequence-dependent fluorescence of the acridine chromophore, the exchange rate of the dye between two DNAs differing by their base composition can be monitored either in the stopped-flow apparatus or in the spectrofluorometer, depending on the rate of this exchange. Capelle et al. (1979) have shown that for slowly dissociating ligands the exchange rate is equal to the off-rate, k_{-1}, from poly[d(A-T)]. The displacement of AcTri2 bound to poly[d(A-T)] has been performed by using a 40-fold excess of sonicated calf thymus DNA. The same results were obtained when displacement was achieved with a 120-fold excess of DNA. DNA was sonicated to avoid the formation of ternary complexes. Furthermore, a low concentration of trimer was used [(AcTri2) = 3×10^{-8} M].

A typical experiment is reported in Figure 7. As for AcTri1 and AcDi, all exchange kinetics fit biexponential processes, suggesting a heterogeneity of binding. Therefore, a mean relaxation time has been defined, according to Schwartz (1968), as the arithmetical mean of the relaxation rates weighted by their respective amplitudes. These values are reported in Table I. The average mean relaxation rates cannot be measured at ionic strength below 0.3 M NaCl because the dissociation rate becomes very small. In these conditions the

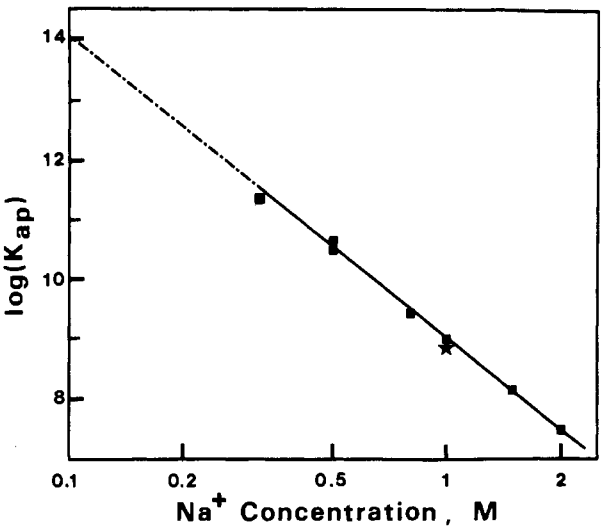

FIGURE 8: Variation of AcTri2 binding constant to poly[d(A-T)] as a function of ionic strength. The full squares correspond to binding constants evaluated from kinetic measurements at various ionic strengths in sodium acetate buffer, pH 5.0, 25 °C. The star corresponds to the binding constant measured in 1 M sodium by competition experiment.

time required to achieve the exchange is too long with respect to the relative unstability of the solution. It should be noted that in 0.3 M NaCl AcTri2 dissociates much more slowly than AcTri1 and even AcDi (1.2×10^{-4} s^{-1} vs. 1.7×10^{-1} s^{-1} and 10^{-2} s^{-1}).

The apparent binding constants to poly[d(A-T)] have been determined by computing the ratios k_1/k_{-1} (Table I). A close agreement is found between the values computed in 1 M NaCl from either competition with ethidium dimer ($K_{app} = 8 \times 10^8$ M^{-1}) or kinetic experiments ($K_{app} = 10^9$ M^{-1}). As shown in Figure 8 a log–log plot of k_1/k_{-1} vs. the ionic strength fits accurately a linear relation, according to Record et al. (1976). These results support the conclusion that the exchange rates measured with sonicated calf thymus DNA actually correspond to the dissociation rate of AcTri2 from poly[d(A-T)]. The slope of this straight line equals $n\psi$, n being the number of interacting charges in the complex and ψ a parameter equal to 0.88 for double-stranded DNA (Record et al., 1976). Consequently the number of ion pairs formed in the interaction of AcTri2 with poly[d(A-T)] is $n = 5.7$, not far from the theoretical maximum of 7 with the fully ionized trimer. In the purpose of comparison an apparent binding constant can be estimated in 0.1 M sodium acetate buffer, pH 5.0, from the preceding linear relationship. This value, $K_{app} = 10^{14}$ M^{-1} (Figure 8), is 5×10^2 times larger than the corresponding constant with AcDi and 3×10^7 times larger than with AcMo (Capelle et al., 1979) and reaches or even goes beyond the DNA affinity of regulatory protein such as *lac* repressor (Riggs et al., 1970) or RNA polymerase (Hinkle & Chamberlin, 1972).

Since all association rates are similar ($k_1 \simeq 2 \times 10^7$ M^{-1} s^{-1}) whether the dye is mono-, di-, or trimeric, the gain in DNA affinity arises from the off-rate constants which show a large decrease on going from the monomer to the trimer. This is an attractive feature since, in the actinomycin series, a slow dissociation rate was shown to be relevant to biological events such as inhibition of transcription (Müller & Crothers, 1968). Furthermore, Mirau & Kearns (1983) have shown tht the slow dissociation rate of actinomycin D from poly[d(G-C)] could be responsible of the inhibition of the B to Z transition of the polymer at very low drug to nucleotide ratio ($r = 1/450$). Now in the series of intercalating 7*H*-pyridocarbazoles there is no

Table II: Binding Affinities (K_{app}) and Efficiencies of Oligomerization (EO) of Acridine Derivatives in 0.1 M Sodium Acetate, pH 5.0

	poly(aminoalkyl) chain[a]		poly(carboxamidoalkyl) chain[b]	
	K_{app} (M^{-1})	EO	K_{app} (M^{-1})	EO
monomer	3×10^6		5×10^4	
dimer	2×10^{11}	0.60	10^7	0.35
trimer	10^{14}	0.46	2×10^7	0.20

[a] The acridine derivatives are AcMo, AcDi and AcTri2, respectively. [b] K_{app} values are from Gaugain et al. (1984). The trimer is AcTri1.

relationship between DNA affinity and the apparition of antitumoral activity (Pelaprat et al., 1981). Differences in the geometry of DNA–dye complexes are usually inferred, but one cannot rule out a priori differences in their kinetic behavior.

In the homogeneous series of acridines linked by poly(aminoalkyl) chains (AcMo, AcDi, and AcTri2), it is clearly shown that the poly[d(A-T)] binding affinity increases with the degree of oligomerization.

The importance of the nature of the linking chain can be pointed out by comparing the efficiency of the oligomerization process for dimers and trimers, built with either poly(aminoalkyl) chains or poly(carboxamidoalkyl) chains, with regard to their DNA binding affinity. The efficiency of the oligomerization process for DNA interaction can be defined by the following ratio:

$$EO = [\Delta G_{obsd}(n) - \Delta G(1)]/[\Delta G_{th}(n) - \Delta G(1)]$$

where $\Delta G_{obsd}(n)$ is the measured free energy of interaction of an n-mer with DNA, $\Delta G(1)$ is the free energy of interaction of the monomer, and $\Delta G_{th}(n)$ is the theoretical free energy of interaction of the n-mer. The latter can be evaluated as follows:

$$\Delta G_{th}(n) = n\Delta G(1) - (n-1)RT \ln (55.6)$$

where the second term accounts for the entropy of mixing. All these free energies of interaction should be calculated in the same conditions (temperature and ionic strength); then

$$EO = \ln [K_{obsd}(n)/K(1)]/\ln [55.6K(1)]^{n-1}$$

where K is the affinity constant of either the monomer [$K(1)$] or the n-mer [$K_{obsd}(n)$].

EO values have been calculated in 0.1 M sodium acetate buffer for AcDi and AcTri2 by comparison with AcMo and for AcTri1 and the corresponding dimer by comparison with the corresponding monomeric [(carboxamidoethyl)amino]-acridine (Gaugain et al., 1984). These values, reported in Table II, show that when a flexible positively charged aminoalkyl chain is used to built dimers or trimers, the gain in DNA affinity vs. the corresponding monomer is very large since respectively 60% and 46% of the theoretical maximum free energy of interaction with DNA are obtained. However, as the number of subunits increases the efficiency decreases likely because of both larger geometric constraints and unfavorable entropic terms due to a strong reduction in degrees of freedom which defavorizes the DNA recognition process. The same feature holds in the carboxamidoalkyl series on going from the dimer (EO = 0.35) to the trimer (EO = 0.20). Nevertheless, it should be pointed out that the efficiency of either dimerization or trimerization via such a chain remains far below that obtained with poly(aminoalkyl) chain (0.35 vs. 0.60 and 0.2 vs. 0.46). This further suggests that the relative rigidity of such an amidated linker prevents the formation of several properly positioned hydrogen bonds between DNA bases and the amide groups of the chain, similar to those that were observed with the corresponding monomer (Gaugain et al., 1981).

Therefore, it appears that an efficient oligomerization requires a positively charged chain, flexible enough to prevent at best the loss in degrees of motional freedom. Furthermore, this last factor likely sets limitations to the oligomerization device, as demonstrated by the progressive decrease of the EO values that occurs when the number of subunits is increased.

Conclusion

This work shows that an acridine trimer (AcTri2) built with a positively charged poly(aminoalkyl) chain, though adopting a folded conformation in solution, is able to tris-intercalate into DNA at a low dye to nucleotide ratio.

This ligand binds to poly[d(A-T)] with a very high affinity and covers six base pairs.

On the one hand, AcTri2 associates to poly[d(A-T)] at a rate similar to those yet reported for other acridine derivatives. Either binding to preexisting kinks in DNA or desolvatation of the dye prior intercalation can be inferred to explain such a phenomenon.

On the other hand, AcTri2 dissociates from poly[d(A-T)] at a dramatically slower rate than the corresponding dimer. A straightforward consequence of this kinetic behavior is an enhanced DNA affinity close to the theory that could be as large as 10^{14} M^{-1} in 0.1 M sodium.

To our knowledge, AcTri2 is the first synthetic ligand that presents such a high affinity for DNA, affording a promising challenge in the field of poly-intercalating antitumoral agents.

Acknowledgments

We thank J. Couprie for her excellent technical assistance and C. Lavayssiere for typing the manuscript.

Supplementary Material Available

Description of the synthesis of AcTri2 and variation of AcTri2 absorbance at 424 nm as a function of pH (Figure S1) (5 pages). Ordering information is given on any current masthead page.

Registry No. **1**, 97613-86-0; **2**, 85185-24-6; **3**, 97613-87-1; **4**, 97613-88-2; **5**, 97613-89-3; **6**, 97633-42-6; **7**, 97613-91-7; **8**, 97613-92-8; AcMo, 59962-52-6; AcDi, 59962-51-5; AcTri1, 97613-93-9; $NH_2CH(CH_2CO_2Et)_2$, 51865-85-1; $PhCH_2OC(O)NH(CH_2)_5NH_2$, 69747-36-0; $HO_2C(CH_2)_2NHBoc$, 3303-84-2; poly[d(A-T)], 26966-61-0; 6-chloro-2-methoxy-9-phenoxyacridine, 7478-26-4.

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DNA Methylation in Mammalian Nuclei†

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ABSTRACT: A novel system to study the methylation of newly synthesized DNA in isolated nuclei was developed. Approximately 2.5% of cytosine residues incorporated into nascent DNA became methylated by endogenous methylase(s), and the level of DNA modification was reduced by methylation inhibitors. DNA synthesis and methylation were dependent on separate cytosol factors. The cytosol factor or factors required for DNA methylation were sensitive to trypsin digestion and were precipitable by $(NH_4)_2SO_4$, suggesting that they were proteinaceous. Time-course experiments revealed a short lag of approximately 20 s between synthesis and methylation in nuclei. The DNAs produced in these nuclei were a mixed population of low molecular weight fragments and higher molecular weight fragments shown to be short extensions of existing replicons. The methylation level found in low molecular weight DNA was lower than that found in bulk L1210 DNA, indicating that further methylation events might take place after ligation of small fragments. These data suggest that newly synthesized DNA is a good substrate for methylase enzymes and that nuclear cytoplasmic interactions may be important in controlling inheritance of methylation patterns.

Alteration of cytosine methylation patterns present in vertebrate DNA has been suggested to be a necessary but not sufficient requirement for altering gene expression (Razin & Riggs, 1980; Doerfler, 1983). The majority of the methylcytosine residues are found in CG sequences (Doskocil & Sorm, 1962), which are underrepresented in eukaryotic DNA (Josse et al., 1961). Hemimethylated sites can be converted into symmetrically methylated sites by "maintenance methylases", thus maintaining the fidelity of the methylated state (Pfeifer et al., 1983).

Studies in many laboratories using inhibitors of 5-methylcytosine formation have shown an inverse correlation between methylation and gene expression (Jones, 1985). This inverse

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