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DNA Bifunctional Intercalators. 1. Synthesis and Conformational Properties of an Ethidium Homodimer and of an Acridine Ethidium Heterodimer†

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ABSTRACT: An ethidium homodimer and an acridine ethidium heterodimer have been synthesized. The ethidium and the acridine chromophore were introduced in such bifunctional intercalators in order to allow the fluorometric study of the interaction of such molecules with DNA, which is reported in the companion paper (Gaugain, B., Barbet, J., Capelle, N., Roques, B. P., & Le Pecq, J. B. (1978) *Biochemistry 17* (following paper in this issue)). In the preparation of the acridine–ethidium dimer, we report the use of acetyl groups as new protecting agents in the phenanthridine series. Conformational studies of these molecules by visible absorption and NMR spectroscopy indicate that these dimers exist in equilibrium between folded and unfolded conformations and that this equilibrium is pH and temperature dependent. Models for the geometry of the folded forms are proposed.

Many DNA intercalating compounds elicit biologically interesting properties and several are used as antitumoral or antiparasitic drugs. It is generally agreed that these properties are related to their reactivity with DNA. In search of more active compounds it is logical to design molecules with the highest possible affinity for DNA. Such a result can be simply obtained using oligomeric derivatives. If each of the subunits could interact with DNA, the binding constant should increase almost exponentially with the number of subunits. Along this line, several bifunctional compounds made up of DNA intercalating dyes (chloroquine, acridine, ellipticine) have recently been prepared (Marquez et al., 1974; Barbet et al., 1975; Canellakis et al., 1976; Delbarre et al., 1976), and several have antitumoral properties (Canellakis et al., 1976; Sinha et al., 1977; unpublished results from our laboratory). Although such molecules can bind to DNA according to different processes, some of them are able to bisintercalate in DNA (Le Pecq et al., 1975; Wakelin et al., 1976). The length and the conformation of the chain linking the two dyes, as well as the chemical nature of the intercalating moieties, have been shown to be of great importance for the interaction with DNA (Le Pecq et al., 1975; Barbet et al., 1976; Canellakis et al., 1976; Fico et al., 1977).

Detailed study of the interaction of these types of molecules with DNA is rendered difficult owing to their high binding affinity. Nevertheless, such a study is necessary if one wants to understand the mechanism of action of such compounds. We were therefore led to synthesize new bifunctional derivatives made up of fluorescent compounds such that the DNA interaction could be more easily studied. Ethidium and acridine were chosen for their well-known DNA intercalating and fluorescent properties (Le Pecq, 1971, 1976, reviews).

In this paper we report the synthesis of an ethidium homodimer "EtDi"[1] and of an acridine ethidium heterodimer "AcEtDi" (Figure 1) and studies of their conformational behavior as a function of pH and temperature by visible and NMR spectroscopies. The following paper details the use of the fluorescent properties of such compounds for studying the DNA binding of bifunctional intercalators (Gaugain et al., 1978).

Experimental Section

The purity of all the compounds was tested by thin-layer chromatography on silica gel with a 8:2:1 mixture of butanol,

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[1] Abbreviations used: EtDi, ethidium dimer; AcEtDi, acridine ethidium dimer; HMDS, hexamethyldisiloxane; Me_2SO (or DMSO in figure), dimethyl sulfoxide.

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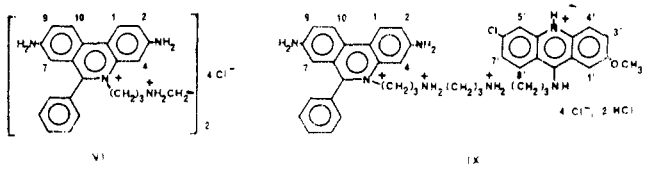

FIGURE 1: Chemical structure of the ethidium homodimer VI and the acridine ethidium heterodimer IX.

trifluoracetic acid, and water as eluent, and their R_f given in brackets.

1. *Synthesis*

3,8-Dicarbethoxyamino-5-(4′,7′-diazaheptyl)-6-phenylphenanthridinium Chloride Dihydrochloride, IV. 3,8-Dicarbethoxyamino-5-(3′-bromopropyl)-6-phenyl phenanthridinium bromide, III (9.6 g, 15.2 mmol) (Watkins, 1952), was suspended in 570 mL of methanol. By addition of 20 mL (0.3 mol) of ethylenediamine, the foregoing salt was dissolved and the solution was refluxed for 6 h. The cooled solution was poured into 75 mL of water. Evaporation of methanol gave orange plates which were washed with water then dried. This pseudo-base was dissolved in 700 mL of ethanol and the solution acidified by 6.3 mL of concentrated hydrochloric acid to yield 3,8-dicarbethoxyamino-5-(4′,7′-diazaheptyl)-6-phenyl phenanthridinium chloride dihydrochloride, IV (5.2 g, 78%), mp 278 °C (R_f 0.60).

Anal. Calcd for $C_{30}H_{38}Cl_3N_5O_4 \cdot 1H_2O$: C, 54.84; H, 6.14; Cl, 16.19; N, 10.66; O, 12.18. Found: C, 54.88; H, 5.79; Cl, 16.25; N, 11.05; O, 11.88.

4,7-Diazadecyl-5,5′-bis(3,8-dicarbethoxyamino-6-hydroxy-6-phenyl-5,6-dihydrophenanthridine), V. Five grams (7.85 mmol) of 3.8-dicarbethoxyamino-5-(4′,7′-diazaheptyl)-6-phenylphenanthridinium chloride dihydrochloride, IV, was suspended in 250 mL of methanol; then the mixture was boiled. Sixteen milliliter of 1 N NaOH was added. The orange solution obtained was treated with 16 mL of 1 N NaOH and 5 g (7.9 mmol) of 3,8-dicarbethoxyamino-5-(3′-bromopropyl)-6-phenylphenanthridinium bromide, III, dissolved in 200 mL of methanol, was added to the solution mixture. After 16 h of refluxing, the solution was cooled and the protected ethidium dimer crystallized as the pseudo-base, V, in yellow plates. It was washed with methanol then dried (2.15 g, 26%; R_f 0.55).

4,7-Diazadecyl-5,5′-bis(3,8-diamino-6-phenylphenanthridium) Dichloride Dihydrochloride, VI = Ethidium Homodimer, EtDi. The pseudo-base, V (2.15 g; 2.06 mmol) was hydrolyzed by heating with 4.8 mL of concentrated sulfuric acid at 130 °C. The cooled mixture was made basic with 19 mL of 10 N ammonia solution to liberate a crude pseudo-base. It was dissolved in 7.5 mL of concentrated hydrochloric acid. By addition of 15 mL of acetone, the ethidium dimer crystallized in purple crystals (1.45 g, 96%), mp 275 °C.

Anal. Calcd for $C_{46}H_{50}Cl_4N_8 \cdot 2CH_3OH \cdot 5H_2O$: C, 57.03; H, 6.78; Cl, 14.03; N, 11.08; O, 11.08. Found: C, 57.67; H, 5.68; Cl, 14.08; N, 11.22; O, 11.20 (R_f 0.09).

3,8-Dicarbethoxyamino-5-(4′,8′,12′-triazadodecyl)-6-phenylphenanthridium Chloride Trihydrochloride, VII. Bis(3-aminopropyl)amine (7.4 mL; 50.9 mmol) was added to a suspension of 3.7 g (5.85 mmol) of 3,8-dicarbethoxyamino-5-(3′-bromopropyl)-6-phenylphenanthridinium bromide, III, in 7.5 mL of methanol. After 5 h of refluxing, the red solution was cooled and poured into water (25 mL). Then methanol was evaporated in vacuo to precipitate the pseudo-base which was washed with water then dried. The orange plates were dissolved in 10 mL of ethanol and the solution was acidified with 1.6 mL of concentrated hydrochloric acid to yield 3,8-dicarbethoxyamino-5-(4′,8′,12′-triazadodecyl)-6-phenylphenanthridinium chloride trihydrochloride, VII (2.85 g, 66%), mp 235 °C (R_f 0.1).

5-(11-(2-Methoxy-6-chloro-9-acridinylamino)-4,8-diazaundecyl)-3,8-dicarbethoxyamino-6-phenylphenanthridinium Chloride Trihydrochloride, VIII. 3,8-Dicarbethoxyamino-5-(4′,8′,12′-triazadodecyl)-6-phenylphenanthridinium chloride trihydrochloride, VII (2.4 g; 3.23 mmol), and 1.3 g (3.88 mmol) of 2-methoxy-6-chloro-9-phenoxyacridine were heated under stirring at 120 °C in 22 g of phenol for 1.5 h. On cooling the mixture solidified. It was dissolved in 11 mL of methanol and the brown solution slowly poured into 360 mL of ether to precipitate 5-(11-(2-methoxy-6-chloro-9-acridinylamino)-4,8-diazaundecyl)-3,8-dicarbethoxyamino-6-phenylphenanthridinium chloride trihydrochloride, VIII, which crystallized from methanol in orange crystals (2.3 g, 73%), mp 272 °C (R_f 0.2).

5-(11-(2-Methoxy-6-chloro-9-acridinylamino)-4,8-diazaundecyl)-3,8-diamino-6-phenylphenanthridinium Chloride Pentahydrochloride, IX = AcEtDi (Method A). The protected heterodimer, VIII (279 mg; 0.28 mmol), was heated under stirring with 0.5 mL of concentrated sulfuric acid for 3 h. The cooled mixture was made basic by the addition of 2.2 mL of 10 N ammonia solution, yielding a "brown gum," which was separated and dissolved in concentrated HCl. This solution was then chromatographed on a column of silica gel, with an 8:2:1 mixture of butanol, trifluoroacetic acid, and water as eluent. The resulting product (R_f 0.1 by thin-layer chromatography on silica gel using above eluent) was evaporated to dryness and dissolved in concentrated HCl. The heterodimer, IX, was then separated as a red oil, by adding acetone to the above mixture. Subsequent addition of ether to this oil produced a solid product, IX, which was filtered and dried in vacuo (30 mg, 12%), mp 265 °C (R_f 0.1).

Anal. Calcd for $C_{42}H_{50}Cl_7N_7O \cdot 4H_2O$: C, 51.00; H, 5.91; Cl, 25.09; N, 9.91; O, 8.09. Found: C, 51.13; H, 5.51; Cl, 23.28; N, 9.83; O, 8.63.

3,8-Diacetamido-6-phenylphenanthridine, X. Acetic anhydride (2.5 mL) was added to 855 mg (3 mmol) of 3,8-diamino-6-phenylphenanthridine, I (Watkins, 1952), in 5 mL of acetic acid. The mixture was stirred at room temperature for 0.5 h and 3,8-diacetamido-6-phenylphenanthridinium acetate precipitated. The suspension was slowly poured into 20 mL of water, and then made basic with 15 mL of concentrated ammonia solution to yield the phenanthridine. It was washed with water, and then dried and crystallized from ethanol in very pale yellow crystals (945 mg, 86%), mp 205 °C (R_f 0.55).

Anal. Calcd for $C_{23}H_{19}N_3O_2 \cdot 1H_2O$: C, 71.30; H, 5.46; N, 10.85; O, 12.39. Found: C, 71.99; H, 5.28; N, 11.02; O, 11.32.

3,8-Diacetamido-5-(3′-bromopropyl)-6-phenylphenanthridinium Bromide, XI. 3,8-Diacetamido-6-phenylphenanthridine, X (500 mg; 1.36 mmol), was refluxed with 50 mL of 1,3-dibromopropane for 6 h. Yellow plates were deposited. After cooling, the mixture was filtered and 3,8-diacetamido-5-(3′-bromopropyl)-6-phenylphenanthridinium bromide, XI, crystallized from methanol in yellow plates (615 mg, 80%), mp 280 °C (R_f 0.45).

Anal. Calcd for $C_{26}H_{25}Br_2N_3O_2 \cdot 1CH_3OH$: C, 53.75; H, 4.84; Br, 26.49; N, 6.96; O, 7.96. Found: C, 53.05; H, 4.62; Br, 26.47; N, 6.80; O, 6.10.

3,8-Diacetamido-5-(4′,8′,12′-triazadodecyl)-6-phenylphenanthridinium Chloride Trihydrochloride, XII. 3,8-Diacetamido-5-(3′-bromopropyl)-6-phenylphenanthridinium

bromide, XI (500 mg; 0.88 mmol), was stirred under reflux with 4 mL (28.4 mmol) of bis(3-aminopropyl)amine in 15 mL of methanol for 6 h. The cooled solution was poured into water and methanol evaporated. A yellow solid was deposited. It was washed with water then dried. This pseudo-base was dissolved in warm ethanol. By acidification with 10 N hydrochloric acid, 3,8-diacetamido-5-(4′-8′,12′-triazadodecyl)-6-phenylphenanthridinium chloride trihydrochloride, XII, precipitated. It was crystallized from methanol as orange plates (385 mg, 64%), mp 252 °C (R_f 0.05).

Anal. Calcd for $C_{32}H_{44}Cl_4N_6O_2 \cdot 5H_2O$: C, 49.49; H, 7.01; Cl, 18.26; N, 10.82; O, 14.42. Found: C, 48.80; H, 6.34; Cl, 17.88; N, 10.28; O, 14.05.

5-(11-(2-Methoxy-6-chloro-9-acridinylamino)-4,8-diazaundecyl)-3,8-diacetamido-6-phenylphenanthridinium Chloride Trihydrochloride, XIII. 3,8-Diacetamido-5-(4′,8′,12′-triazadodecyl)-6-phenylphenanthridinium chloride trihydrochloride, XII (274 mg; 0.4 mmol), and 161 mg (0.48 mmol) of 2-methoxy-6-chloro-9-phenoxyacridine were heated under stirring at 120 °C in 1 g of phenol for 2 h. On cooling the mixture solidified. After dissolution in 2 mL of methanol, the resulting brown solution was poured into 100 mL of ether. 5-(11-(2-Methoxy-6-chloro-9-acridinylamino)-4,8-diazaundecyl)-3,8-diacetamido-6-phenylphenanthridinium chloride trihydrochloride, XIII, precipitated. It was then filtered, washed with ether, and crystallized from methanol in brown-orange prisms (327 mg, 88%), mp 260 °C (R_f 0.15).

Anal. Calcd for $C_{46}H_{52}Cl_5N_7O_3 \cdot 4H_2O$: C, 55.23; H, 6.05; Cl, 17.72; N, 9.80; O, 11.20. Found: C, 55.31; H, 6.15; Cl, 16.29; N, 9.21; O, 11.22.

5-(11-(2-Methoxy-6-chloro-9-acridinylamino)-4,8-diazaundecyl)-3,8-diamino-6-phenylphenanthridinium Chloride Pentahydrochloride, IX = AcEtDi (Method B). 5-(11-(2-Methoxy-6-chloro-9-acridinylamino)-4,8-diazaundecyl)-3,8-diacetamido-6-phenylphenanthridinium chloride trihydrochloride, XIII (562 mg; 0.61 mmol), was suspended in 28 mL of 10% HCl–methanol. The mixture was refluxed under stirring for 2 h. The cooled solution was filtered; then the solvents were removed by evaporation in vacuo for 4 h to give 5-(11-(2-methoxy-6-chloro-9-acridinylamino)-4,8-diazaundecyl)-3,8-diamino-6-phenylphenanthridinium chloride pentahydrochloride, IX, as a purple power (436 mg, 79%), mp 265 °C (R_f 0.1).

Anal. Calcd for $C_{42}H_{50}Cl_7N_7O \cdot 4H_2O$: C, 51.00; H, 5.91; Cl, 25.09; N, 9.91; O, 8.09. Found: C, 50.84; H, 5.78; Cl, 23.42; N, 10.00; O, 8.55.

2. *NMR Studies*

^{1}H NMR spectra were recorded at 270 MHz with a Bruker WH 270 spectrometer operating on the Fourier transform mode and locked to the deuterium resonance of solvent D_2O. Probe temperatures were regulated by a Bruker B ST 100/700 controller and monitored by observing the splitting in ethylene glycol. Chemical shifts (δ) were measured from an external reference made up of a capillary filled with a solution of 5% hexamethyldisiloxane (HMDS) in CCl_4. δ in ppm are reliable to ±0.01 ppm.

In order to reduce the intensity of the HOD solvent resonance, the spectra, at concentration $\geq 10^{-3}$ M, were recorded with a strong and continuous irradiation of the HOD signal. For lower concentrations, standard homonuclear gated decoupling has been used.

Solutions were made in pure D_2O at different pD values which were determined with a Tacussel TC 60/N pH meter according to the equation, pD = meter reading + 0.4 (Glasoe & Long, 1960). The concentrations of the stock solutions were measured by spectrophotometric analysis. For each study and at each concentration, all the ^{1}H signals were assigned from double resonance experiments.

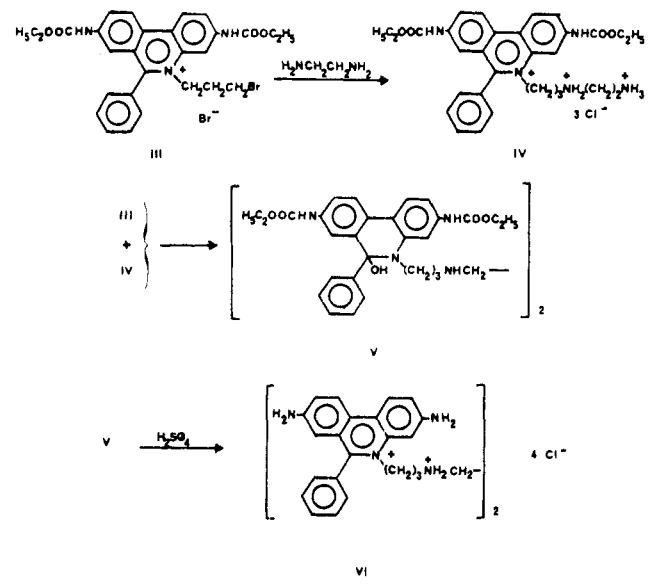

FIGURE 2: Scheme of EtDi synthesis.

3. *Visible Spectra*

Visible spectra of AcEtDi and of the parent compounds XIV and XV have been recorded on a Varian Techtron 635 UV-visible spectrometer at concentration of 10^{-4} M in 0.1 M acetate buffer (pH 5). Studies of the AcEtDi and EtDi visible spectra as a function of pH were monitored at 5×10^{-5} M (25 °C) in 0.05 M NaCl solutions.

Results and Discussion

I. *Synthesis*

1. *Ethidium Homodimer "EtDi" VI.* 4,7-Diazadecyl-5,5′-bis(3,8-diamino-6-phenylphenanthridinium) dichloride dihydrochloride, VI (ethidium homodimer "EtDi"), has been synthesized according to the scheme in Figure 2; 3,8-dicarbethoxyamino-5-(3′-bromopropyl)-6-phenylphenanthridinium bromide, III, has been prepared according to Watkins (1952). By reaction with ethylenediamine, this quaternary salt gives 3,8-dicarbethoxyamino-5-(4′,7′-diazaheptyl)-6-phenylphenanthridinium chloride dihydrochloride, IV. This compound is allowed to react with the bromopropyl quaternary compound III leading to 4,7-diazadecyl-5,5′-bis(3,8-dicarbethoxyamino-6-hydroxy-6-phenyl-5,6-dihydrophenanthridine), V, the pseudo-base of the protected ethidium homodimer. This intermediate is not purified but is immediately reacted with sulfuric acid. The protective carbethoxy groups are then hydrolyzed. After neutralization, the ethidium homodimer VI is crystallized. The structure of EtDi has been established previously, by NMR experiments (Roques et al., 1976).

Attempts to synthesize other ethidium homodimers, with different chain lengths, have still been unsuccessful. Attempts for nucleophilic displacement of the bromine atom of III by compounds similar to IV but with longer alkylamino chains have led to decomposition with the recovering of some phenylphenanthridine. On the other hand, the dimer cannot be obtained directly by reaction of an excess of III with the diamine as for the preparation of acridine dimers (Barbet et al., 1975).

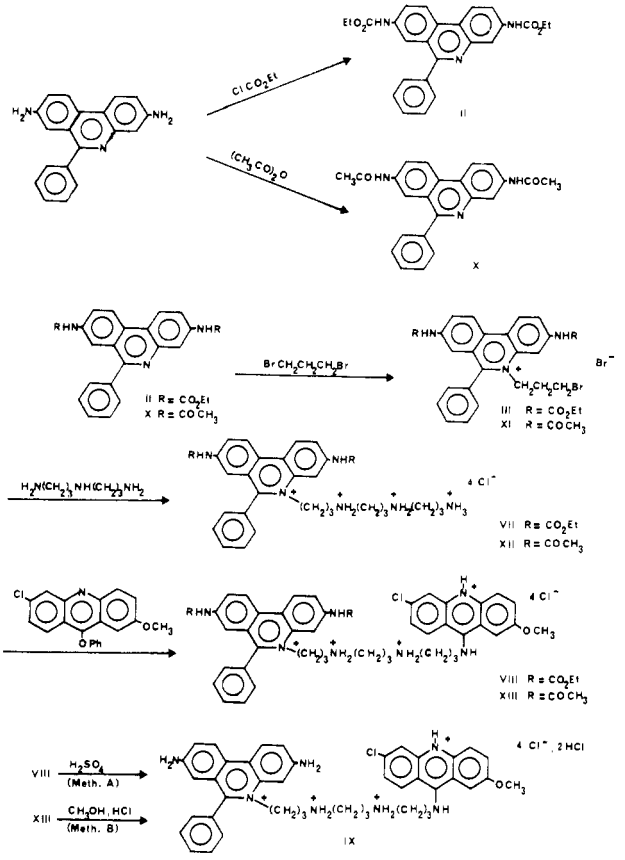

FIGURE 3: Scheme of AcEtDi synthesis.

2. *Acridine Ethidium Heterodimer "AcEtDi"*. 5-(11-(2-Methoxy-6-chloro-9-aminoacridinyl)-4,8-diazaundecyl)-3,8-diamino-6-phenylphenanthridinium chloride pentahydrochloride, IX, has been synthesized according to the scheme presented in Figure 3; 3,8-diamino-6-phenylphenanthridine, I, prepared according to Watkins (1952) reacts with acetic anhydride to give 3,8-diacetamido-6-phenylphenanthridine, X, which is quaternized by 1,3-dibromopropane; 3,8-diacetamido-5-(3′-bromopropyl)-6-phenylphenanthridinium bromide, XI, thus obtained is allowed to react with an excess of bis(3-aminopropyl)amine to give 3,8-diacetamido-5-(4′,8′,12′-triazadodecyl)-6-phenylphenanthridinium chloride trihydrochloride, XII. This compound reacts with 2-methoxy-6-chloro-9-phenoxyacridine to afford the protected AcEtDi, XIII. When refluxed in methanol–HCl according to Walls & Whittaker (1952), the acetamido groups are rapidly hydrolyzed and AcEtDi is obtained in good yield and can be used without further purification.

The best way for the synthesis of such an heterodimer AcEtDi is to start from the aminoalkylphenanthridinium XII according to the above scheme (Figure 3). We have attempted, alternatively, to effect the heterodimer synthesis beginning with aminoalkylacridine monomers, which can be prepared by use of a considerable excess of polyamine. However, spontaneous dimerization occurs both during purification and during storage at room temperature. All attempts to make these compounds react with halogenoalkyl derivatives have failed and the corresponding acridine homodimer is obtained as the major product.

As for EtDi, preparation of AcEtDi requires the previous protection of ethidium amino groups. In a first assay, the carbethoxy group has been used like a protective agent as described by Watkins (1952) and Walls (1947). Removal of these

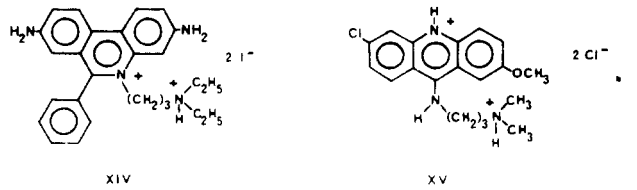

FIGURE 4: 3,8-Diamino-5-(3′-diethylaminopropyl)-6-phenylphenanthridinium iodide hydroiodide, XIV, and 2-methoxy-6-chloro-9-(3′-dimethylaminopropylamino)acridinium chloride hydrochloride, XV, used as parent compounds in visible and NMR studies.

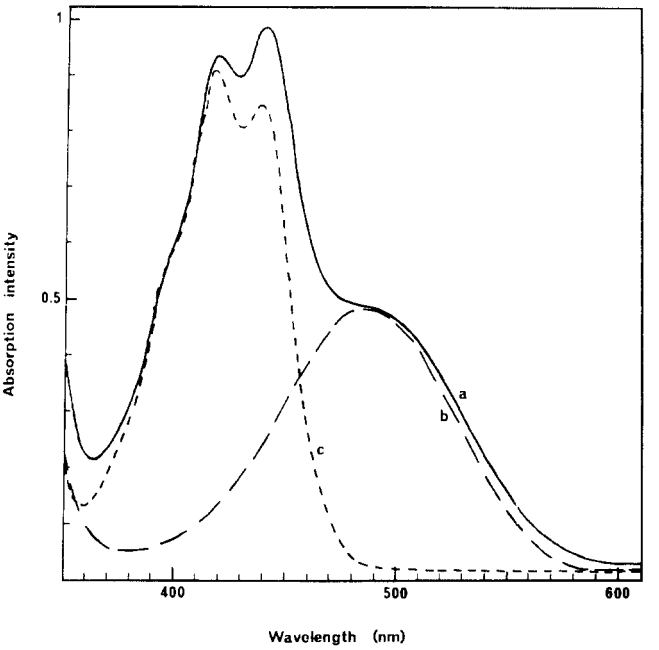

FIGURE 5: Visible spectra in 0.1 M acetate buffer (pH 5) of (a) AcEtDi, (b) phenanthridinium compounds XIV, and (c) acridinium derivative XV. Concentration was 9.0×10^{-5} M for each compound.

groups requires the use of hot concentrated sulfuric acid. Because of the sensitivity of the 9-aminoacridine toward strong acidic or alkaline medium (Albert & Ritchie, 1943), in this method, the yield of the last step is poor (12%). Therefore, more easily removable protective agents must be used. Unfortunately, protection of the amino groups of the phenanthridine I with formyl or carbobenzyloxy groups inhibits the quaternization by 1,3-dibromopropane. However, a good yield is obtained with acetyl protecting groups removable in relatively mild conditions (Walls & Whittaker, 1952).

The structure of AcEtDi has been established by visible and NMR experiments. The visible spectrum of AcEtDi was compared with those of the parent compounds: 3,8-diamino-5-(3′-diethylaminopropyl)-6-phenylphenanthridinium iodide hydroiodide, XIV, and 2-methoxy-6-chloro-9-(3′-diethylaminopropylamino)acridinium chloride hydrochloride, XV (Figure 4). Such compounds XIV and XV containing an aminoalkyl chain are more suitable, because it has been shown that substitution by charged chains appreciably modifies the spectroscopic properties of the chromophore (Le Bret & Chalvet, 1977). Visible spectrum of AcEtDi (Figure 5) exhibits all the characteristic absorption bands of XIV and XV. The NMR spectrum of AcEtDi in Me_2SO-d_6 solution is reported in Figure 6. Assignment of all resonances was made by comparison with XIV and XV and with the aid of double-resonance experiments. The ^1H NMR spectrum of XV has been previously interpreted (Barbet et al., 1976).

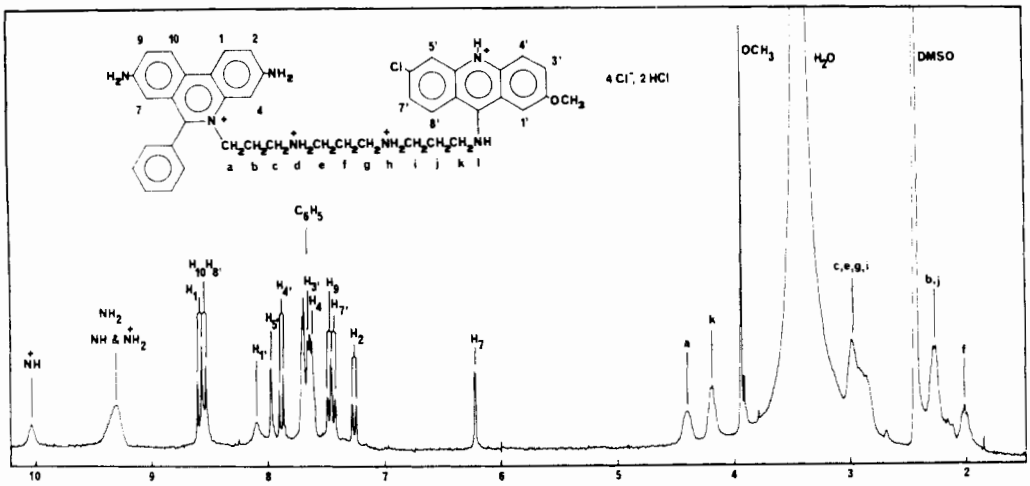

FIGURE 6: ^{1}H NMR spectrum of the acridine ethidium heterodimer at 20 °C (10^{-2} M) in Me_2SO-d_6; HMDS was used as an internal reference.

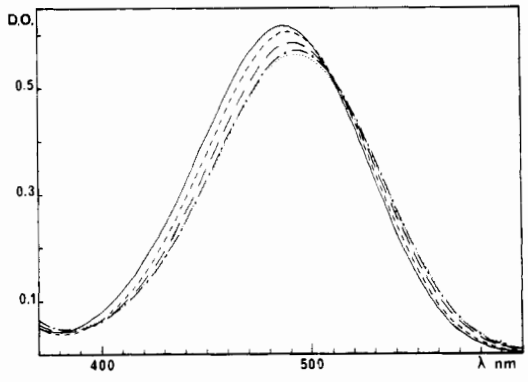

FIGURE 7: Visible spectrum of ethidium dimer (0.5×10^{-4} M) in 0.05 M NaCl for different values of pH. (—) pH 3.0; (- - -) pH 4.2; (— —) pH 4.9; (— · —) pH 5.8; (· · ·) pH 7.4.

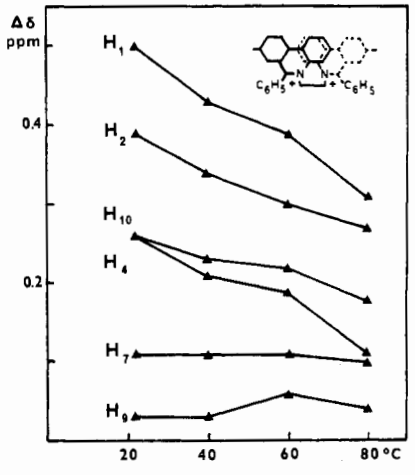

FIGURE 8: Temperature dependence of the chemical shifts of the aromatic protons of EtDi (10^{-3} M) as compared with those of phenanthridinium monomer XIV (10^{-3} M), at pH 7. $\Delta\delta = \delta_{XIV} - \delta_{EtDi}$.

II. *Conformational Studies of EtDi and AcEtDi*

Similar to acridine dimers (Barbet et al., 1976), both EtDi and AcEtDi can exist in equilibrium between folded and unfolded conformations. Such behavior could be of great importance for the DNA intercalation process. Before any DNA interaction study, it was necessary to analyze the conformational states of the present dimers in aqueous solution at different pH.

1. *Ethidium Dimer.* Since the ethidium dimer is a bisquaternary ammonium compound, its ionization will not be strongly affected by varying the pH of its aqueous solution. However, the visible spectrum of EtDi shows a red shift as a function of pH and also hypochromism (Figure 7), whereas no such behavior is observed in the phenanthridinium monomer XIV. This can be easily explained by the titration of the ammonium charges of the chain linking the two rings, which decreases the electrostatic repulsion between the ammonium groups of the chain and the quaternized rings. This effect leads to an important increase of self-stacked dimers in accordance with the observed hypochromism. Indeed, Warshaw & Tinoco (1966) have shown that strong stacking interactions, as in dinucleotides, generally lead to hypochromism.

In order to confirm the presence of such intramolecular stacked structures, we have performed NMR studies in 10^{-3} M D_2O solutions. At this concentration, intermolecular interactions both for ethidium monomer and dimer are minimized as already reported (Roques et al., 1976). At 21 °C and pH 7, a comparison of the NMR spectra of EtDi and the corresponding monomer XIV shows a strong upfield shift for the H_1, H_2, H_4, and H_{10} protons in the dimer (Figure 8). This shielding effect is about twice that observed at pH 3. With respect to these results, it can be concluded that EtDi is in equilibrium between folded and unfolded conformations in accordance with the results of the visible study. This equilibrium can be monitored as a function of temperature between 21 and 80 °C at pH 7 where the folded form is expected to be preponderant (Figure 8). On raising the temperature, a marked deshielding is observed for the H_1, H_2, H_4, and H_{10} protons of EtDi as compared with similar protons of XIV in the same conditions. In contrast, the less shielded protons H_7 and H_9 are practically unaffected. Because the strongest shielding is observed for the H_1, H_2, H_4, and H_{10} protons as compared with the H_7 and H_9 protons, a model for the folded form (Figure 8) can be proposed using ring current calculations made by Giessner-Prettre & Pullman (personal communication) on ethidium bromide.

2. *Acridine Ethidium Dimer.* As for EtDi, the visible spectrum of the acridine ethidium dimer shows an important hypochromism on the acridine band on raising the pH, whereas a moderate hypochromism and a weak red shift appear for the phenanthridinium band (Figure 9). The curves of the absorption intensity as a function of pH at fixed wavelengths clearly shows the presence of two titrations (Figure 10). The first titration (low pH) can be monitored by changes in either

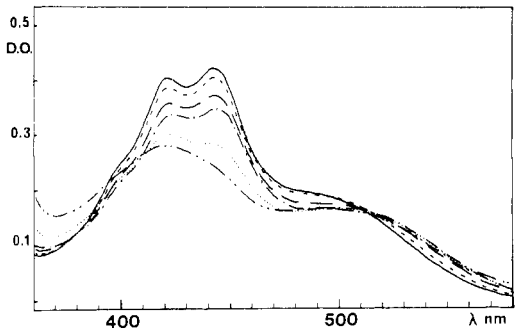

FIGURE 9: Visible spectrum of acridine ethidium dimer (0.5×10^{-4} M) in 0.05 M NaCl for different values of pH. (—) pH 3.0; (- - -) pH 5.1; (— —) pH 6.0; (— · —) pH 6.6; (· · ·) pH 8.0; (— · · —) pH 9.5.

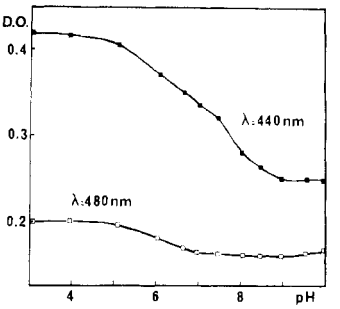

FIGURE 10: Absorption intensity of acridine ethidium dimer (0.5×10^{-4} M) in 0.05 M NaCl at λ = 440 nm (-■-) and λ = 480 nm (-□-) as a function of pH.

the acridine or the phenanthridinium absorption bands, whereas the second titration is observed only when monitoring changes in the acridine absorption. The first titration ($pK \sim 6.0$) probably corresponds to successive deprotonations of the ammonium charges on the chain linking the two rings, leading to an increase of the stacked dimer population (similar to that observed for EtDi). The second titration ($pK = 7.9$) is easily identified as the deprotonation of the intracyclic nitrogen atom of the acridine. This is readily apparent by comparison with the visible spectrum of the acridine monomer XV, in which the pK of the intracyclic nitrogen is found equal to 8.2.

Due to the overlap of the acridinium and phenanthridinium chromophores in the visible spectrum, we have further investigated the pH dependence of the AcEtDi stacking interaction by NMR. The assignments of the resonances of all the protons, except $H_{1'}$, $H_{3'}$, and $H_{4'}$ which are superimposed, were established by comparison with XIV and XV and double-resonance experiments (Figure 11). The methylene signals of the aminoalkyl chain at 4.1 ppm (a) and 3.9 ppm (k) have been assigned to the CH_2 linked to the phenanthridinium ring and the 9-acridine amino group, respectively, in accordance with their position in the plane of the aromatic ring and the vicinity of the charged phenanthridinium nitrogen for the former. On the other hand, the more shielded signal at 1.8 ppm has been attributed to the CH_2 group (f) located at the center of the chain and far from the two aromatic rings. From these assignments, all of the other CH_2 signals have been assigned by double-resonance (Figure 11).

At pH 7, 21 °C, and at low AcEtDi concentration (5×10^{-4} M), several of the aromatic protons (H_1, H_2, H_4, H_7, $H_{5'}$, and $H_{8'}$) of AcEtDi are observed shifted upfield by 0.3 to 0.6 ppm, as compared with identical protons in the NMR spectra of the monomeric compounds XIV and XV. The induced shielding of these protons on AcEtDi readily suggest the presence of some folded structure in the dimer. It must be emphasized that at this concentration (10^{-3} to 10^{-4} M AcEtDi), intermolecular dimer-dimer interactions are negligible as observed in previous NMR studies of EtDi (Roques et al., 1976). Furthermore, the aromatic resonances which are shielded in the AcEtDi NMR spectrum at pH 7 and at 21 °C (H_1, H_2, H_4, H_7, $H_{5'}$, and $H_{8'}$) undergo slight downfield shifts (≤0.1 ppm; data not shown) as the temperature is raised to 80 °C, providing further evidence for the presence of a folded ⇌ unfolded dimer equilibrium in solution. However, the small magnitude of these temperature induced shifts (≤0.1 ppm) suggests that a folded configuration still predominates at pH 7, even at higher temperatures.

Variations in the chemical shifts of all the assigned protons of AcEtDi at 10^{-3} M are presented as a function of pH in Figure 12. Pronounced upfield shifts of 0.1–0.5 ppm are observed upon increasing pH, for protons on the methylene chain (Figure 12A) and on the two aromatic chromophores (Figure 12B). These results are consistent with a folded dimer conformation at high pH, which results in increased proton shielding due to stacking of the two aromatic chromophores. The shapes of the titration curves (Figure 12) indicate that the largest induced chemical shift changes occur between pH 5.5 and pH 9. Deprotonation of both the acridine ring ($pK \sim 8$, Figure 10) and the chain amino groups ($pK \sim 6$, Figure 10) directly influences the intramolecular stacking interactions between aromatic rings, as observed by the induced chemical shift changes of the AcEtDi protons (Figure 12). On the phenanthridinium ring, the largest induced upfield shifts with increasing pH (pH 5.5–9) are observed for the H_1, H_2, H_4, and H_7 protons ($\Delta\delta$ = 0.3–0.4 ppm), whereas the H_{10} and H_9 protons are shifted upfield by only 0.17 and 0.06 ppm, respectively. On the acridinium ring, the resonances from the methoxy group and those of the $H_{1'}$, $H_{3'}$, and $H_{4'}$ protons (data not shown) are weakly shielded ($\Delta\delta$ = 0.05 ppm), whereas the $H_{8'}$, $H_{7'}$, and $H_{5'}$ proton resonances are strongly upfield shifted by 0.3–0.4 ppm (Figure 12B). This internal comparison between protons located on each side of the two heterocyclic moieties leads us to propose a folded conformation for AcEtDi with partial overlap between the two chromophores, analogous to that proposed for EtDi. In such a model, the portion of the acridine ring carrying the methoxy group would not be stacked over the phenanthridinium ring (in agreement with the above induced chemical shift results), thus decreasing steric hindrance with the 6-phenyl substituent.

Conclusion

Two bifunctional intercalators EtDi and AcEtDi made up of highly fluorescent heterocycles (phenanthridine and acridine) have been synthesized. In the series of acridine dimers, we have shown (Barbet et al., 1976) that such bifunctional compounds are able to bisintercalate in DNA by the so called "excluded site" model (Bauer & Vinograd, 1970). Such a binding process requires that the distance between the two aromatic rings is >10.1 Å. In EtDi and AcEtDi the lengths of the chains linking the two chromophores, computed from model building data, are 11.4 Å for EtDi and 13.5 Å for AcEtDi. Despite this apparent agreement with the required distance, EtDi and AcEtDi do not bisintercalate in DNA (Gaugain et al., 1978). However, it can be observed that, for the present dimers, these maximal distances between the two rings are calculated with the linking chains in their more extended conformations. Due to the presence of the 6-phenyl ring on the phenanthridinium part of these dimers, such extended forms of the chains are unfavorable because this configuration enhances the steric hindrance between the phenyl ring and several

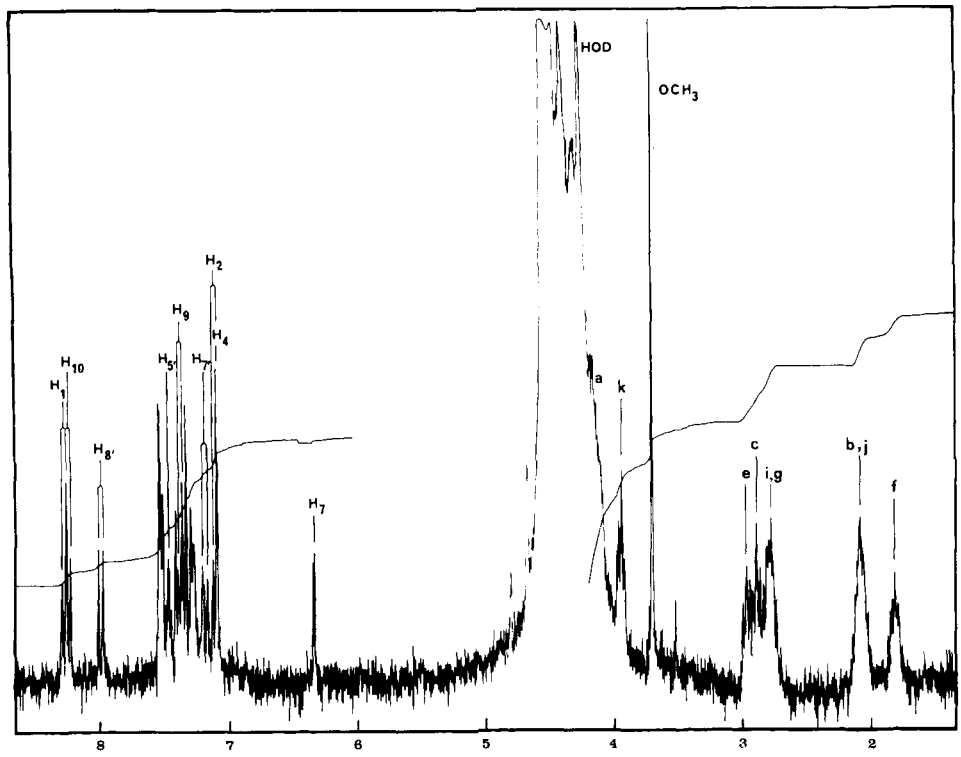

FIGURE 11: ^1H NMR spectrum of AcEtDi (10^{-3} M) in D_2O at pH 3.

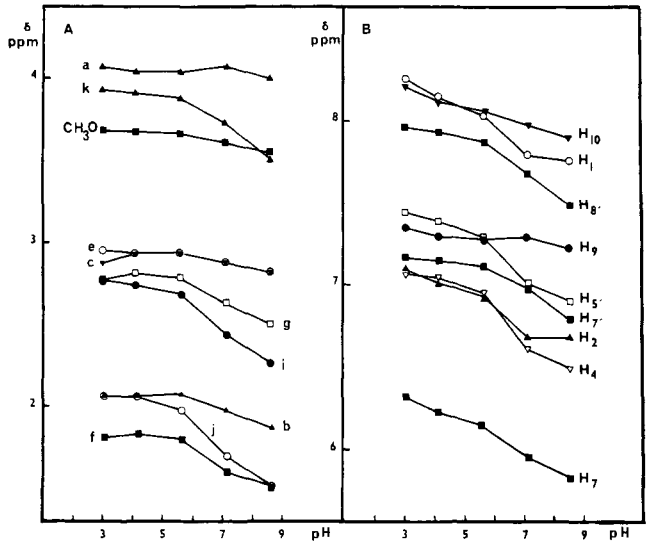

FIGURE 12: Induced chemical shift changes of the AcEtDi (10^{-3} M) protons as a function of pH.

methylene groups of the chain decreasing their degree of freedom. In order to reduce such an unfavorable entropy factor, the extended conformation of the linking chain must adopt a more diagonal orientation to join the two chromophores. Such a structural requirement decreases the maximal distance between the two aromatic rings and so prevents their DNA bisintercalation (Gaugain et al., 1978).

In aqueous solutions EtDi and AcEtDi are in equilibrium between folded and unfolded conformations. As shown by visible and NMR studies, this equilibrium is pH and temperature dependent. However, in comparison with the intramolecular stacking of the acridine dimers (Barbet et al., 1976), the stacked conformations of EtDi and AcEtDi still predominate even at high temperature (80 °C, pH 7) owing to the greater stability of their folded forms. The proposed geometry for these folded forms are in accordance with a diagonal orientation of the connecting chain and partial overlap of the aromatic chromophores.

It is of considerable importance to recognize that the DNA binding of either EtDi or AcEtDi by a bisintercalation process will require opening of the folded drug forms (Le Pecq et al., 1975). Therefore, the DNA binding free energy of these bifunctional drugs is directly dependent on the strength of their intramolecular stacking interactions. Consequently, it appears that at physiological conditions (pH ~ 7, 37 °C) the most appropriate bifunctional compounds should be those which retain the highest proportion of unfolded forms in order to exhibit stronger pharmacological DNA-binding properties. Such assumptions have been confirmed in preliminary experiments on these and other derivatives synthesized in our laboratories.

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References

Albert, A., & Ritchie, B. (1943) *J. Chem. Soc.*, 458.

Barbet, J., Roques, B. P., & Le Pecq, J. B. (1975) *C. R. Acad. Sci., Ser. D*, 851.

Barbet, J., Roques, B. P., Combrisson, S., & Le Pecq, J. B. (1976) *Biochemistry 15*, 2642.

Bauer, W., & Vinograd, J. (1970) *J. Mol. Biol. 47*, 419.

Canellakis, E. S., Schaw, Y. H., Hanners, W. E., & Schwartz, R. A. (1976) *Biochim. Biophys. Acta 418*, 277.

Delbarre, A., Roques, B. P., & Le Pecq, J. B. (1976) *C. R. Acad. Sci., Ser. D, 284*, 81.

Fico, R. M., Teh-Kuei-Chen, & Canellakis, E. S. (1977) *Science 198*, 53.

Gaugain, B., Barbet, J., Capelle, N., Roques, B. P., & Le Pecq, J. B. (1978) *Biochemistry 17* (following paper in this

issue).
Glasoe, P. K., & Long, F. A. (1960) *J. Phys. Chem. 64,* 188.
Le Bret, M., & Chalvet, O. (1977) *J. Mol. Struct. 37,* 299.
Le Pecq, J. B. (1971) in *Methods of Biochemical Analysis* (Glick, D., Ed.) Vol. 20, pp 41-86, Wiley, New York, N.Y.
Le Pecq, J. B. (1976) in *Biochemical Fluorescence Concepts* (Chen, R. F., & Edelhoch, H., Eds.) Vol. II, pp 711-736, Marcel Dekker, New York, N.Y.
Le Pecq, J. B., Le Bret, M., Barbet, J., & Roques, B. P. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72,* 2915.
Marquez, V. E., Cranston, J. W., Ruddon, R. W., & Burckhalter, J. H. (1974) *J. Med. Chem. 17,* 856.
Roques, B. P., Barbet, J., & Le Pecq, J. B. (1976) *C. R. Acad. Sci., Ser. D 283,* 1453.
Sinha, B. K., Philen, R. M., Sato, R., & Cysyk, R. L. (1977) *J. Med. Chem. 20,* 1528.
Wakelin, L. P. G., Romanos, M., Canellakis, E. S., & Waring, M. J. (1976) *Stud. Biophys. 60,* 111.
Walls, L. P. (1947) *J. Chem. Soc.,* 67.
Walls, L. P., & Whittaker, N. (1952) *U.S. 2,* 594, 265, April 22.
Warshaw, M. M., & Tinoco, J., Jr. (1966) *J. Mol. Biol. 20,* 29.
Watkins, T. I. (1952) *J. Chem. Soc.,* 3059.

DNA Bifunctional Intercalators. 2. Fluorescence Properties and DNA Binding Interaction of an Ethidium Homodimer and an Acridine Ethidium Heterodimer†

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Appendix: Numerical Solution of McGhee and von Hippel Equations for Competing Ligands

Marc Le Bret#

ABSTRACT: An ethidium homodimer and an acridine ethidium heterodimer have been synthesized (Gaugain, B., Barbet, J., Oberlin, R., Roques, B. P., & Le Pecq, J. B. (1978) *Biochemistry 17* (preceding paper in this issue)). The binding of these molecules to DNA has been studied. We show that these dimers intercalate only one of their chromophores in DNA. At high salt concentration ($Na^+ > 1$ M) only a single type of DNA-binding site exists. Binding affinity constants can then be measured directly using the Mc Ghee & Von Hippel treatment (Mc Ghee, J. D., & Von Hippel, P. H. (1974) *J. Mol. Biol. 86,* 469). In these conditions the dimers cover four base pairs when bound to DNA. Binding affinities have been deduced from competition experiments in 0.2 M Na^+ and are in agreement with the extrapolated values determined from direct DNA-binding measurements at high ionic strength. As expected, the intrinsic binding constant of these dimers is considerably larger than the affinity of the monomer (ethidium dimer $K = 2 \times 10^8$ M^{-1}; ethidium bromide $K = 1.5 \times 10^5$ M^{-1} in 0.2 M Na^+). The fluorescence properties of these molecules have also been studied. The efficiency of the energy transfer from the acridine to the phenanthridinium chromophore, in the acridine ethidium heterodimer when bound to DNA, depends on the square of the AT base pair content. The large increase of fluorescence on binding to DNA combined with a high affinity constant for nucleic acids makes these molecules extremely useful as nucleic acid fluorescent probes. In particular, such molecules can be used in competition experiments to determine the DNA binding constant of ligands of high binding affinity such as bifunctional intercalators.

The biological properties and the antitumoral activity of bifunctional intercalators (Fico et al., 1977; Sinha et al., 1977; unpublished results from our laboratory) are thought to be related to their high DNA binding affinity (Le Pecq et al., 1975). It is therefore of interest to characterize and understand in detail the DNA binding of such molecules. Their interaction with DNA is complex. Several different types of binding are involved and in some cases bisintercalation occurs (Le Pecq et al., 1975; Wakelin et al., 1976).

Because the DNA binding affinity of such molecules is very high, a direct measurement of this binding affinity is partic-

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