(in a stepped gradient of 2:1, followed by 9:1) to obtain 948 mg (50%) of the title compound as a pale yellow powder, mp 144.5–151 °C. Anal. ($C_{22}H_{25}N_5O_2S$) C, H, N, S.

RPAR-Synovitis Technique.[1] Lewis rats were dosed orally with drug or placebo 1 h prior to iv administration of bovine serum albumin (BSA) in 0.2 mL of pyrogen-free saline, followed by the intraarticular injection of 0.54 mg of rabbit anti-BSA antibody in 0.03 mL of pyrogen-free saline in one knee, and saline alone in the contralateral joint, all under light anesthesia. After 3 h the rat was again dosed orally with drug or placebo. The drug dose used in calculations was the total administered both before and after lesion induction.

About 17 h after lesion induction, the animal was killed and both knee joints were exposed. The subpatellar areolar tissue, with attendant synovium, was excised and weighed. Differences between the weight of antibody- and saline-injected knees, "Δ synovial weight", were considered to represent the inflammatory response for each animal. Differences in "Δ synovial weight" between lesion controls and drug-treated rats (five animals per treatment group) are presented as the mean percent inhibition. For compounds producing greater than 20% inhibition, the reported value is statistically different from control by an analysis of variance.

Adjuvant-Induced Arthritis in Rats (AAR).[7] **(a) Prophylactic Regimen.** Heat-killed *Mycobacterium tuberculosis* (from the Ministry of Agriculture, Fisheries and Food Central Veterinary Laboratory, Weybridge, Surrey, England) was prepared by grinding to a fine powder. It was then weighed, mixed with paraffin oil (6 mg/mL), and homogenized.

The animals were dosed with drug 1 h prior to challenge with adjuvant and then for 21 consecutive days. Control animals were given methylcellulose. Injection of 0.1 mL of the adjuvant was made into the left hind paw. The left and right hand paw volumes were measured immediately on a plethysmograph. Final measurements were taken on both paws on day 21 of the assay. Data were reported as "Δ paw volume".

(b) Therapeutic Regimen. The same procedure was followed except that the lesion was allowed to develop for 7 days before the first drug treatment. Treatment was continued for 35 days. Treatment of controls and measurement of "Δ paw volume" were conducted as described in (a) above.

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Dynamics and Thermodynamics of the Counterion Effect in a 7*H*-Pyridocarbazole Dimer (Ditercalinium). Hypothesis of a Nonbisintercalative Binding Mode to Calf Thymus DNA at High Drug/Base Ratio

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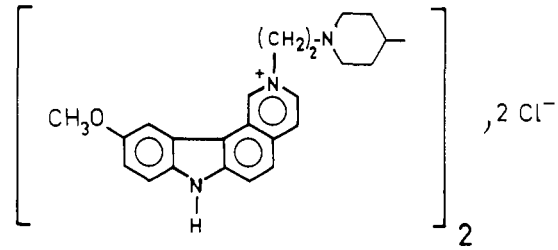
Ditercalinium, a 7*H*-pyridocarbazole dimer designed to bisintercalate into DNA, forms tight ion pairs in water with inorganic and organic anions. The thermodynamics and kinetics of the acetate–ditercalinium pairing has been investigated by means of T-jump spectroscopy. The formation of the pair has a constant estimated to 1000 M^{-1} and proceeds via a fast two-step mechanism with a relaxation time of 12 μs (acetate pH 5) to 50 μs (cacodylate, pH 7.5) involving an intermediate solvent-separated ion pair. A strong association of ditercalinium to cardiolipid has been observed and is expected to be involved in the respiratory chain inhibition induced by ditercalinium (unpublished results). Direct estimates of the binding constants of the drug to calf thymus DNA were obtained by means of UV titrations at high drug/base ratio (>0.17). The maximum number of binding sites per base both at pH 5 and pH 7.5 was found to be 0.22, a value consistent with monointercalation as expected from the prediction of Shafer's model for the interaction of bifunctional ligands to DNA. This work also supports the hypothesis that significant ionic binding may account for the ditercalinium/DNA interaction at high base/drug ratios (0.2).

Intercalation into DNA of planar aromatic molecules such as phenantridines, acridines, ellipticines, etc. is thought to be the primary act (if not the significant) that accounts for the cytotoxicity and eventual antitumor properties of these compounds. To this respect and in order to potentiate the affinity and specificity of their monofunctional counterparts, numerous bifunctional derivatives consisting of two intercalative moieties linked together by an aliphatic or alicyclic chain have been synthesized and tested against various experimental tumors.[1] One of this compounds, a 7*H*-pyridocarbazole dimer known under the generic name of ditercalinium (2,2′-([4,4′-bipiperidine]-1,1′-diyldi-2,1-ethanediyl)bis[10-methoxy-7*H*-pyrido[4,3-*c*]carbazole]),[2,3] specially designed to be a bisintercalator, showed promising antitumor activity (NSC366241) and had been introduced in phase 1 clinical trial until it proved to be highly hepatotoxic (possibly as a strong inhibitor of the electron-transport chain in the mitochondrion inner membrane[4,5] and was hence withdrawn from human test.



The numerous experimental data gained on DNA/ditercalinium interactions through various methods (ethidium binding competition, viscosimetry, NMR spectroscopy, electron microscopy) were interpreted in terms of the monointercalation of ditercalinium into DNA at physiological pH ($K_a = 10^7$ M^{-1}, pH 7.4) and of its bisin-

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tercalation at pH 5 with a marked increase of the binding affinity ($K_a = 5 \times 10^8$ M^{-1}).[6-12]

These conclusions lead to several comments that shall be presented and developed in this account. (i) The enhancement of the affinity at low pH suggests the occurrence of a significant ionic contribution to the overall binding process and leads to the question as to know whether this ionic interaction is only a step toward further formation of a final intercalative or nonintercalative hydrophobic complex, or if ditercalinium, as being polycationic at low pH, may interact with DNA just as other tri- or tetravalent cations, as spermine and spermidine,[13] namely in a purely ionic way. (ii) The pH effect between 7.4 and 5 on the binding constant shows that the nitrogen atoms of the linking chain are being protonated in this pH range. This means that the basicities of the nitrogen atoms in the piperidine rings are considerably weakened with respect to usual pK values (around 9) found in aliphatic or alicyclic nitrogen derivatives.[14] As a consequence, the actual state of protonation of the nitrogen atoms at the pHs of the reported studies is not known (nor is the hydrophobic character and the hydrogen bonding capability of the linking chain) although it should constitute a relevant piece of information in the general understanding of ditercalinium/DNA interactions in terms of hydrogen bond formation and Coulombic effects. (iii) The polycationic ditercalinium is expected to strongly interact with anions present in the buffers in the "in vitro" investigations or encountered "in vivo" by the drug. Indeed, anions proved to induce dramatic spectral changes (this report) which are similar to those observed in the binding of the drug to DNA. It must be stressed that anion effects were not considered in the previous studies although they may lead to deep perturbations of DNA morphology which would make questionable some of the experimental results.

We shall now report, in a first step, the determination of the acid–base properties of ditercalinium (the measurement of pKs of the piperidine ring nitrogen atoms), and the investigation of the binding of the polycationic drug to some of the inorganic anions usually found in buffers (acetate, chloride, phosphate, cacodylate). Then, with the conclusions of this preliminary study in mind, understanding the mechanism of DNA/ditercalinium interaction will be attempted.

T-jump relaxation spectroscopy was used to study the binding of the drug to anions and to DNA. The time-dependent concentrations of free and bound drug was monitored by the variations of the absorbance at 263 nm where ditercalinium presents a strong absorption (ϵ = 71 000 at λ = 263 nm).[10] The definite advantage of equilibrium perturbation methods as compared to the irreversible methods like stopped-flow is that, (i) they are able to detect fast processes, (ii) they may lead to estimates of the reversed kinetic constant which is a measure of the residence time of the drug in its binding sites, this parameter being relevant to the therapeutic properties of the compound, and, finally, (iii) they allow, in favorable cases, the determination of the thermodynamics of the equilibrium through the analysis of the relaxation amplitudes.

Materials and Methods

Chemicals. Ditercalinium hydrochloride was a generous gift from Laboratoire Roger Bellon (batch PM III 264). Solutions of ditercalinium chloride at a concentration of 0.87 mM were prepared by dissolving weighted amounts of the compound in water. Solutions had to be warmed at 40 °C; no subsequent precipitation occurred. Aliquots were introduced in buffer solutions to obtained final concentrations of 1–70 μmol/L. It was checked that, even at a concentration of 70 μM, no deviation from the Beer law was observed. In this concentration range, dramatic spectral effects were induced when certain anions such as phosphate, chloride, perchlorate, anionic surfactants below and above their cmc, and anionic phospholipids (among them the cardiolipid) were introduced even at concentrations in the tens of millimolar range (see Results and Discussion). The presence of these anions could hence be misleading when estimating the binding of the drug to DNA, and consequently their use should be prohibited. Acetate (pH 5), cacodylate, and Tris (pH 7.5) proved to be only slightly disturbing (see Results and Discussion) and were used both in the thermodynamic and kinetic investigations.

Calf thymus DNA (highly polymerized sodium salt) was purchased from Sigma Chemical Co. It was dissolved in 0.01 M acetate buffer, pH 5, and sonicated for about 8 min at 4 °C in order to reduce the viscosity. DNA concentrations were expressed with respect to nucleotides with ϵ at 260 nm equal to 6600 M^{-1} cm^{-1}.

Polyadenylic acid (poly A, Sigma) was dissolved in 10 mM sodium cacodylate. The concentrations in poly A were estimated with ϵ = 10 000 M^{-1} cm^{-1} per base at 257 nm.

Spectrophotometry. Absorbance spectra were recorded with a Cary 118 spectrophotometer equipped with 3-mL quartz cuvettes. Fluorescence of ditercalinium proved to be too weak to be confidently used to monitor drug concentrations.

Perturbation Kinetics. The T-jump relaxation spectrometer was previously described.[15] The amplitude of the spectrophotometric detection signal of 10 mV corresponds to a variation of 1 m O.D. unit of the absorbance of the solution. The time constant of the heating was 2 μs (0.001 μ F capacitor) or 10 μs (0.005 μ F capacitor), and the amplitude of the temperature jump was 1.5 and 7 °C, respectively.

Potentiometric Measurements of the pKs of Ditercalinium. The acid–base properties of ditercalinium, in the absence of any spectral changes in the pH range in between 9 and 4, were investigated by titration of a 8.7×10^{-4} M ditercalinium chloride in water at 25 °C by nitric acid and potassium hydroxide, with a combined glass electrode. The electrode potential was measured with a Tacussel millivoltmeter. It was observed that at a nitrate concentration of 1 mM irreversible precipitation of the drug occurred regardless of the pH. Experimental data were processed by a general fitting procedure described elsewhere.[16]

Transient Electric Dichroism (TED). The temperature-jump spectrometer was used to induce electric field alignment of DNA. The light from a 100-W HBO lamp was polarized with a Polaroid 12345 UV polarizer. The ionic strength of the solutions was 5 mM and the applied field ranged 10–18 kV/cm. At low optical densities, the difference of absorbance in the presence and without an applied static electric field (electric dichroism) of light polarized with an angle α with respect to the direction of the field is

$$A = \tfrac{3}{2} A_i F (\overline{\cos^2 \Theta} - \tfrac{1}{3})(\cos^2 \alpha - \tfrac{1}{3})$$

where A_i is the isotropic absorbance and Θ the angle between E

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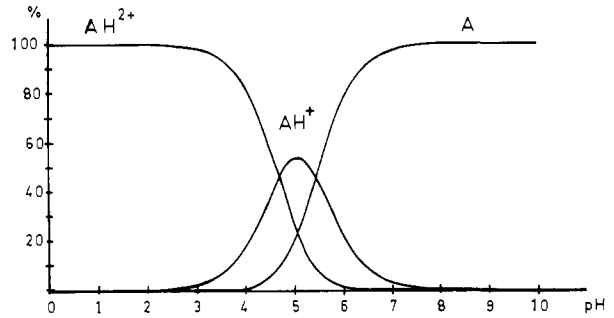

Figure 1. Relative populations of the ionic forms of ditercalinium as a function at pH. A is the quaternized dication, and AH^+ and AH^{2+} are the mono- and diprotonated species, respectively (pK_1 = 4.7 ± 0.2, pK_2 = 5.5 ± 0.2).

and the transition dipole moment responsible for the optical transition; $\overline{\cos^2 \theta}$ is the mean value on all possible orientations and is $^1/_3$ when θ is 55°. F is an orientation factor which depends on the field strength, on the length of DNA rods and their electric dipole moments, and on the ionic strength of the solution. For a given value of the angle α, the sign of the dichroism depends on θ and readily allows discrimination between intercalators (θ > 55°) and groove binders (θ < 55°).[17-19]

Results and Discussion

Acid-Base Properties of Ditercalinium. The potentiometric titration of ditercalinium leads to two close acidity constants of 4.7 ± 0.2 and 5.5 ± 0.2, readily assigned to protonation of the nitrogen atoms of the piperidine rings. The large decrease of basicity as compared to that usually expected for aliphatic or alicyclic nitrogen atoms[14] is likely to arise from the inductive effect of the neighboring quarternized nitrogen atoms on the pyridocarbazole rings.[14] For the same reason, protonation of one of the nitrogen atoms of the linking chain causes a decrease of pK of the other. Hence at pH = 7.2 ditercalinium is essentially dicationic whereas at a pH around 5 it is a mixture of di-, tri-, and tetracationic species. The average number of positive charges of the molecule at this pH would be around 3 (Figure 1).

Interaction of Buffer Anions with Ditercalinium. The absorption spectrum of ditercalinium at pH 5 is significantly altered in the presence of chloride, phosphate, perchlorate, sodium dodecyl sulfate under its cmc (5×10^{-4} M at 0 ionic strength) and above (in contrast, the neutral surfactant Triton X100 induces no effect), nitrate, anionic phospholipids such as the cardiolipid, irrespective to the ionic strength of the medium. Acetate has only a slight but significant effect (spectra 1 and 2 in Figure 2). When increasing amounts of chloride, perchlorate, or phosphate are added at pH 5, a spectral red-shift builds up, resulting in an isosbestic set of spectra (see Figure 2, where only the effect of chloride is shown). The absorbance at 300 nm of the limit spectrum is approximately the half of the initial optical density at this wavelength. With time a decrease of the absorbance is observed in chloride and phosphate solutions (whereas 10 or 100 mM acetate solutions are stable) which leads to the loss of the isosbestic point at 320 nm. This latter process, is likely to arise from microaggregations and microprecipitations of ditercalinium under the influence of the anions as previously observed with other DNA binding drugs.[20]

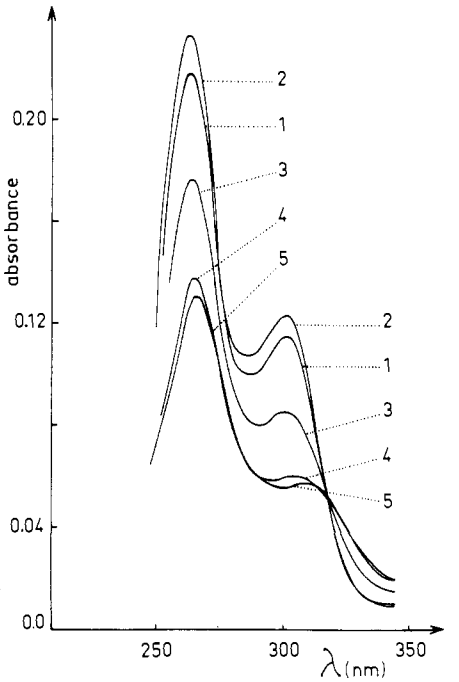

Figure 2. Titration of ditercalinium (2.9×10^{-6} M) by NaCl; (1) acetate, pH 5, 100 mM, 0. NaCl; (2) acetate, pH 5, 10 mM, 0. NaCl; (3) acetate, pH 5, 10 mM, 2.97×10^{-2} M NaCl; (4) acetate, pH 5, 10 mM, 5.88×10^{-2} M NaCl; (5) acetate, pH 5, 10 mM, 1.06×10^{-1} M NaCl.

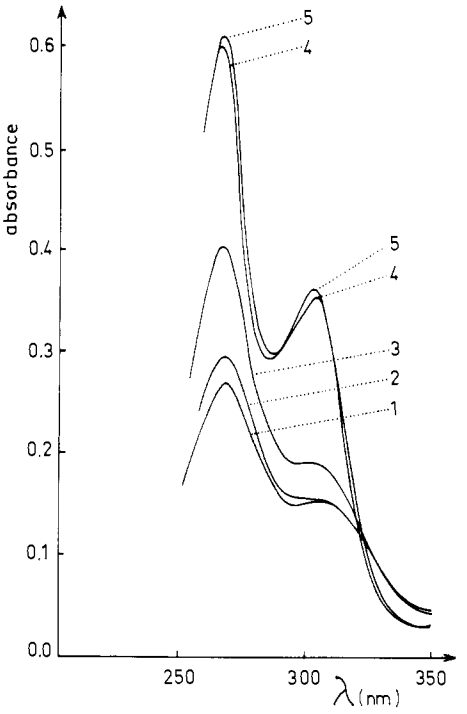

Figure 3. UV spectra of ditercalinium chloride (8.6×10^{-6} M) in water/dioxane mixtures. The values of electric constants are from ref 22. (1) 1% H_2O, ϵ = 2.1; (2) 5% H_2O, ϵ = 3.73; (3) 10% H_2O, ϵ = 5.71; (4) 20% H_2O, ϵ = 11; (5) 30% H_2O, ϵ = 18.2.

When ditercalinium is introduced in aprotic dipolar solvent like dimethylformamide and dimethyl sulfoxide the absorbance spectrum is similar to that recorded in 10 mM acetate buffer. However, the same amounts of drug in dioxane, an aprotic apolar solvent (the dielectric con-

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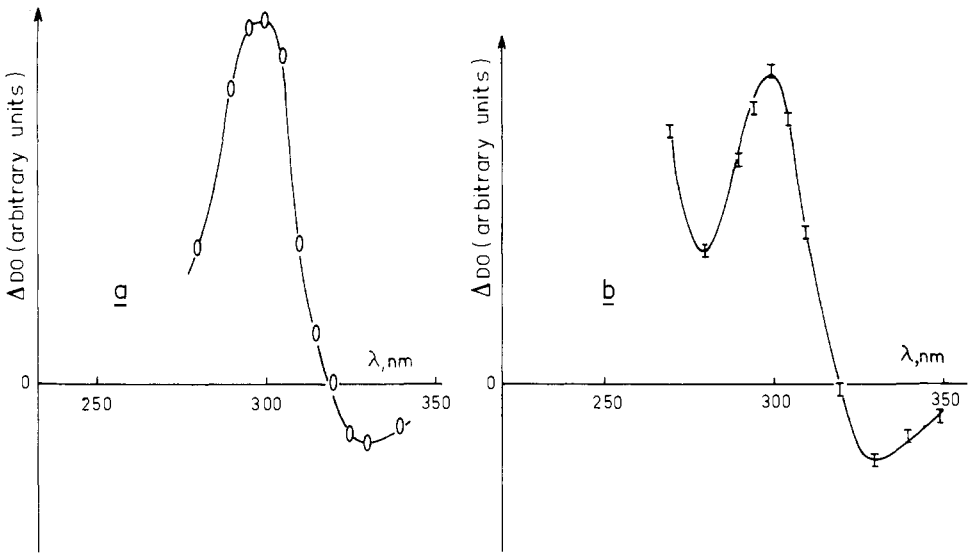

Figure 4. (a) Amplitude of T-jump relaxation of ditercalinium in acetate, 100 mM, pH 5, as a function of the observation wavelength; (b) difference between the spectra of free ditercalinium and ditercalinium (pair spectra 1 and 5 in Figure 2).

stant is 2.1), and in water/dioxane mixtures of varying dielectric constants show the typical spectral change previously induced by chloride in water with a comparable ratio of the absorbances at 300 nm between the limit spectra (Figure 3). At 20% water the dielectric constant is 11 and the limit spectrum similar to that recorded in polar solvents is reached.[21] According to the Kirkwood theory of association between charged or dipolar entities,[22] the equilibrium constant is a function of a solvent parameter defined as $(\epsilon - 1)/(2\epsilon + 1)$. When the parameter reaches the limit value $^1/_2$, the binding constant becomes K_i. It is worth noting that the limit spectrum in Figure 3 corresponds to a value of the parameter close to 0.5.

This is good evidence for the electrostatic character of the interaction leading to the red-shifted spectrum of the complex. It can be hence postulated that, in dioxane, ditercalinium chloride exists as an ion pair. In water in the presence of anions like chloride, ion pairs are also formed though less easily but to an extent that can be large if the anion concentrations is large as compared to that of the ligand. In order to get insights into the thermodynamics and the kinetics of the ion-pair formation in water, fast T-jump relaxation can be profitably utilized.

Ditercalinium in pH 5 Acetate Buffer. As mentioned above, solutions of ditercalinium in 100 mM acetate, cacodylate, and Tris are suitable for this investigation since they are stable and present, although to a lower degree than chloride solutions, the spectral variation which is evidence for ion-pair formation. The fact than the acetate–ditercalinium ion pair formation constant is seemingly much lower than that of the chloride–ditercalinium pair is an advantage in T-jump experiments since it ensures satisfactorily large relaxation amplitudes (see eq 2).

When a 40 μM ditercalinium chloride solution in 100 mM acetate buffer pH 5 is submitted to a fast temperature jump of 1.5 °C a rapid and large (5×10^{-3} O.D. unit/°C), monoexponential relaxation phase is observed. The relaxation time of the equilibriation process is 12 ± 2 μs and depends neither on ditercalinium concentrations in the range investigated (10–70 μM) nor in acetate concentrations between 25 and 100 mM. The relaxation can be unambiguously assigned to the equilibrium involving the formation of the ion pair on the ground of the strict similarity of the variation of the relaxation amplitude with the observation wavelength and the difference spectrum between the free and paired chromophore; in particular, zero amplitude at 320 nm corresponds to the isosbestic point of the spectra (Figure 4). The temperature elevation favors the dissociation of the pair (negative formation enthalpy).

Kinetics of Ion-Pair Formation. The lack of dependence of the relaxation time on substrate and buffer concentrations in the ranges investigated confirms that dimerization of the drug by intermolecular stacking does not occur. This conclusion, readily predictable from the cationic nature of the drug, is further substantiated by the fact that intermolecular association should lead to a linear dependence of the square of the relaxation time with drug concentration. The constant value of the relaxation time throughout the range of reactant concentrations indicates that the observed phenomenon results from a monomolecular equilibrium. Intramolecular chromophore stacking can be ruled out due to the rigid linking chain and to the presence of the positive charges which prevent folding of the molecule. Hence, the relaxation is assigned to the exchange between a loose, solvent separated and a tight ion pair.

The general kinetic scheme for the association pathway is

$$S + A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} P_L \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} P_T$$

where S is a cationic site, A the buffer anion, P_L solvent-separated ion pair, and P_T a tight ion pair.

This pathway is associated with two relaxation times:

$$\tau_1^{-1} = k_1[\bar{S} + \bar{A}] + k_{-1}$$

$$\tau_2^{-1} = k_2 \frac{\bar{S} + \bar{A}}{[\bar{S} + \bar{A}] + K_L^{-1}} + k_{-2} \qquad (1)$$

where K_L is the formation constant of the solvent-separated pair. The relaxation phase associated with the time τ_1 is expected not to be detectable because of the lack of spectral modification between free and loosely bound ditercalinium and, besides, the time is too fast, the reaction being diffusion-controlled.

In order that the expression of τ_2, the relaxation time of the observable phase, not depend on the ionic concentrations (as observed), one must have $K_L \gg$ [ions]. On the

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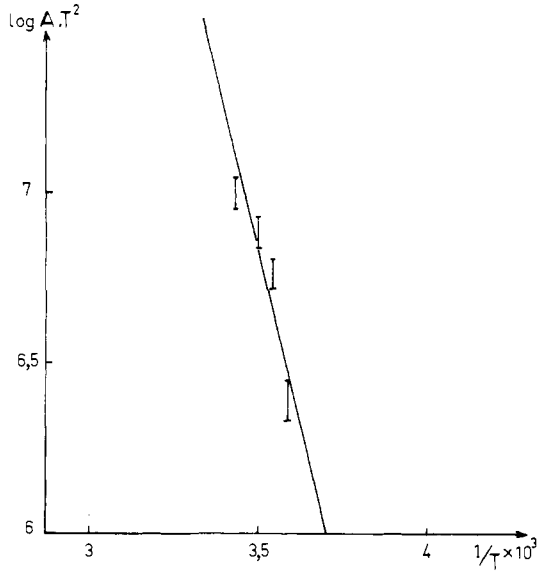

Figure 5. Variation of the product of the relaxation amplitude by T^2 (see eq 3) as a function of $1/T$ ditercalinium chloride ± 4.15×10^{-5} M; ΔT = 1.5 °C, λ = 300 nm.

grounds of the analysis of the thermodynamics of ion-pair formation given below, this hypothesis will be substantiated.

Thermodynamics of Ion-Pair Formation. The relaxation amplitude per degree, $\Delta A/\Delta T$, is readily related to the spectral and thermodynamic properties of the system by

$$\frac{\Delta A}{\Delta T} = C_0 l \frac{\Delta\epsilon_{T\text{-}L} \times K_{app}}{(1 + K_{app})^2 RT^2}\left[\Delta H_{T\text{-}L} + \frac{\Delta H_L}{K_L \bar{A} + 1}\right]$$

The equilibrium constants are defined as

$$K_{app} = \bar{P}_T/[\bar{S} + \bar{P}]$$

$$K_L = \bar{P}_L[\bar{S} \times \bar{A}] \text{ and } K_T = \bar{P}_T/\bar{P}_L$$

C_0 is the overall drug concentration, $\Delta\epsilon_{T\text{-}L}$ is the spectral difference at wavelength λ, and ΔH^* is the overall enthalpy of pair formation at fixed anion concentrations.

When K_{app} is significantly larger than unity (which is likely to be the case since the ion pair is stable), this expression simplifies to

$$\frac{\Delta A}{\Delta T} = C_0 \frac{\Delta\epsilon_{T\text{-}L}}{K_{app}} \frac{\Delta H^*}{RT^2} \text{ with } \Delta H^* = \left[\Delta H_{T\text{-}L} + \frac{\Delta H_L}{K_L \bar{A} + 1}\right] \quad (2)$$

Separate values of K_{app} and ΔH^* which appear as their ratio in eq 2, can be obtained by running T-jump experiments at different starting temperatures and constant ΔT. If ΔH^* does not depend on temperature in the range of investigation, an estimate of ΔH^* is provided by the plot of the amplitude according to

$$\log\left(\frac{\Delta A}{\Delta T} T^2\right) = \frac{\Delta H^*}{R} \frac{1}{T} + \text{constant} \quad (3)$$

ΔH^* is estimated to be −8 ± 1.5 kcal/mol (Figure 5) and K_{app} to be 6 ± 1 when taking $\Delta\epsilon_{T\text{-}L}$ equal to 18 500 at 300 nm, ΔT, the amplitude of the temperature jump, equal to 1.5 °C, $C_0 = 4.2 \times 10^{-5}$ M, and T = 20 °C. The negative value of the enthalpy is consistent with the observation of decreasing stability of the pair with increasing temperature.

If it is assumed that (i) the formation of the tight pair is essentially entropy driven (due to the strong entropy increase which results from the loss of organization of the water layer around the ions), the large enthalpy would mainly be that of the loose pair formation, and that (ii) the decrease of entropy of this latter process (loss of translational degrees of freedom) is relatively small, the equilibrium constant for the formation of the loose pair, K_L, would be large enough to justify the hypothesis which has led to eq 1. The equilibrium constant of the overall binding process, K_{ov}, is readily related to K_L and K_T

$$K_{ov} = \bar{P}_T/[\bar{S} \times \bar{A}] = K_L K_T = K_{app} K_L$$

When, as postulated above, $K_L \gg [\bar{A} + \bar{S}]$, hence, $K_{app} = K_T = k_2/k_{-2}$. Estimating the value of K_{ov} to be around 1000 as inferred from the observation that sodium cations bind polyanionic entities with that affinity,[23] the mean lifetime of the ion pair, defined as $1/k_{-2}$ lies in the 1 ms range (with $\tau_2^{-1} = k_2 + k_{-2} = 12$ μs).

Ditercalinium at Neutral pH. Ditercalinium introduced in cacodylate (100 mM, pH 7.5) shows a decrease of the absorbance at 300 nm and a slight increase at 330 nm with respect to 10 mM acetate solutions which we readily assigned to pair formation. The spectral changes are larger than those observed in 100 mM acetate buffers and suggest the presence of larger amounts of associated ions.

The T-jump investigation shows essentially similar features as in acetate buffers (strict similarity between the difference spectrum and the variations of relaxation amplitude with wavelength, independent of the relaxation time with ion concentrations) although the relaxation time is significantly slower (50 ± 5 μs).

The increase of the overall ion pair formation constant together with the decrease of the relaxation rate is understood in terms of a decrease of the reaction rate constant k_{-2} in eq 1 (with k_2 left unchanged). It is worth stressing that the increase of ionic associations is an intrinsic property of the cacodylate ions rather than an effect of acidity of the medium since at pH 7.2 the linking chain is uncharged.

Interaction of Ditercalinium with Calf Thymus DNA. Spectral Effect of the Drug/DNA Association. In 10 or 100 mM pH 5 acetate solutions, increasing amounts of DNA added to a 3 μM drug solution induce spectral changes proportional to DNA concentrations. When the drug/DNA ratio reaches the value of 0.22 ± 0.03, further introduction of large amounts of DNA produces a weak steady decrease of the absorption with loss of the isosbestic point due, probably, to drug precipitation resulting from its neutralization (see also the Experimental Section on ditercalinium binding to poly A) (Figure 6). Although experiments performed at 200 mM acetate (pH 5) did not show any significant departure from this behavior, the 100 mM buffer was preferably used to minimize ditercalinium/acetate ion pair formation. Interestingly, titration of a 1.9 μM solution of ditercalinium in acetate buffer 100 mM (Figure 7) by increasing amounts of poly A induces a change in the absorbance of the drug similar to that observed with anions and DNA. At a concentration of poly A of 10.35 μM (spectrum 4 on Figure 7), no precipitation occurs. When poly A concentrations reach the value of 14 μM, loss of the isosbestic point occurs as a consequence of a precipitation, at a drug/base ratio in between 0.14 and 0.19. The quantitative titration of ditercalinium is consistent with a very large affinity constant. These conclusions are identical with those reported for DNA (see above) and point to binding mechanisms similar,

(23) Zoltewicz, J. A.; Servando, M. *J. Phys. Chem.* **1986**, *90*, 5820.

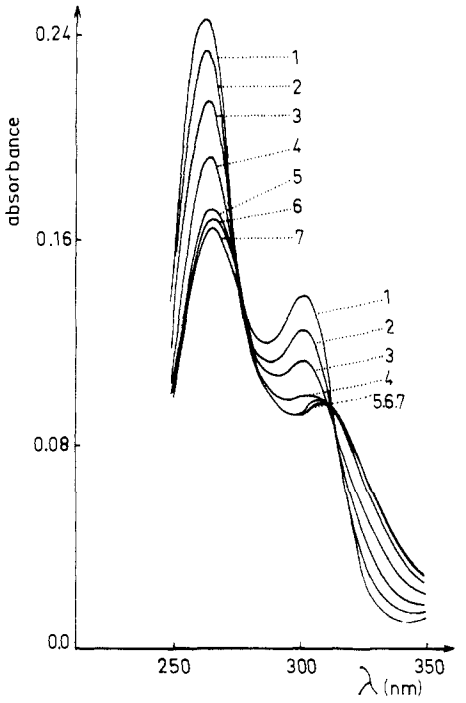

Figure 6. Titration of ditercalinium chloride (2.9×10^{-6} M) by calf thymus DNA in acetate, 10 mM, pH 5; (1) [DNA] = 0; (2) 3.33×10^{-6} M; (3) 6.66×10^{-6} M; (4) 1.11×10^{-5} M; (5) 1.35×10^{-5} M; (6) 2.21×10^{-5} M; (7) 3.3×10^{-5}. Saturation is obtained at ditercalinium/DNA = 0.22 (spectrum 5).

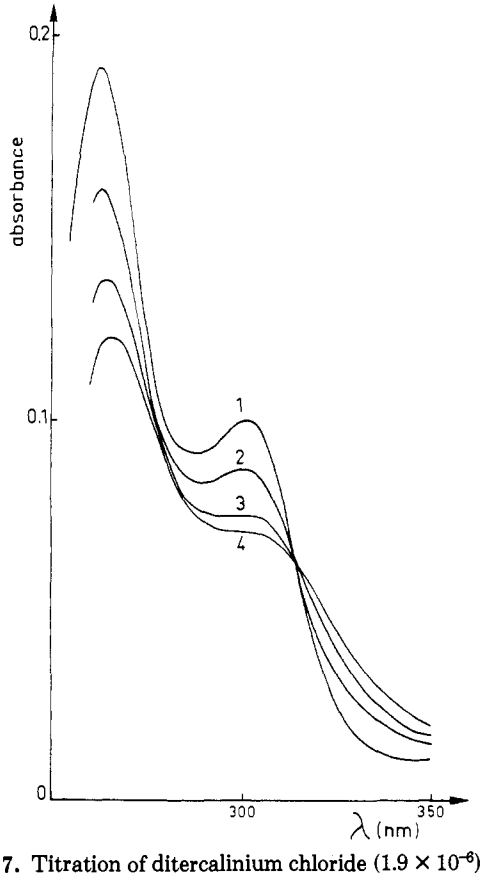

Figure 7. Titration of ditercalinium chloride (1.9×10^{-6}) by poly A in acetate buffer, 100 mM, pH 5; (1) poly A = 0, (2) 3.45×10^{-6} M, (3) 6.9×10^{-6} M; (4) 10.35×10^{-6} M.

at least to some extent, in both cases.

The relevant conclusions of these observations are the following. The drug is stoichiometrically titrated by DNA; this shows that the affinity constant is large. Full titration necessitates a DNA concentration 4 times as large as that of the drug, meaning a maximum number of binding site per base of 0.22 (1 mol of the chromophore covers approximately four bases), a value close to that found for most DNA-interacting drugs. This indicates that, under the prevailing experimental conditions (high drug/DNA ratio), ditercalinium does not bisintercalate since, if it were the case, r max should be around 0.1.[7,8] This observation is consistent with the model proposed by Shafer to account for the binding of bisintercalators at high (0.2) drug/base ratio. According to this model, the bifunctional drug should bisintercalate at ratio less than 0.1 and monointercalate when D/B = 0.2.[24,25] Attempts to explore the low region of the ratio by titrating fixed amounts of binding sites by increasing drug concentrations failed at low drug since the amounts of unbound drug are too vanishingly small for a significant use of the law of mass action as expressed in the Scatchard form. When D/B reaches 0.17, rough estimates of the binding constants at pH 5 ($K_a = 5 \pm 3 \times 10^8$ M^{-1}) and at pH 7.5 ($K_a = 2$ (± 1) $\times 10^7$ M^{-1}) are determined from Scatchard plots (Figures 8 and 9). The ethidium/ditercalinium competition techniques has permitted investigation of the binding at low ratio and has led to a value of r_{max} compatible with bisintercalation.[7,8] However, somewhat surprisingly, some experiments performed at D/B found in the range of our own study (see the last point in Figure 1B in ref 7 which corresponds to log $(1 + 2r) = 1.5$ and hence $r = 0.2$, which, nevertheless, fits the linear plot, hence indicating no change in the binding mode all over the D/B range) were also taken as evidence for bisintercalation.

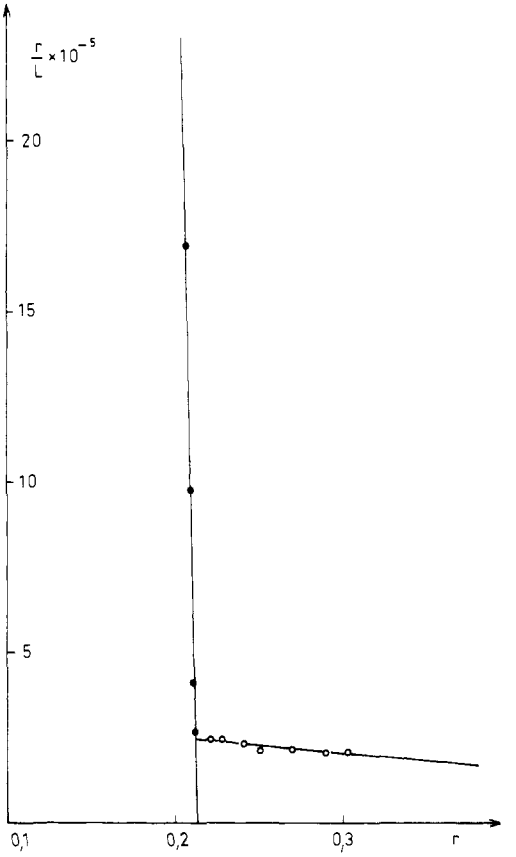

Figure 8. Titration of calf thymus DNA (6.94×10^{-6} M) in acetate, 10 mM, pH 5, by ditercalinium chloride. The limit absorbance ratio, V, is 0.6 at $\lambda = 262$ nm.

(24) Shafer, R. H. *Biopolymers* **1980**, *19*, 419.
(25) Shafer, R. H.; Wating, M. J. *Biopolymers* **1980**, *19*, 431.

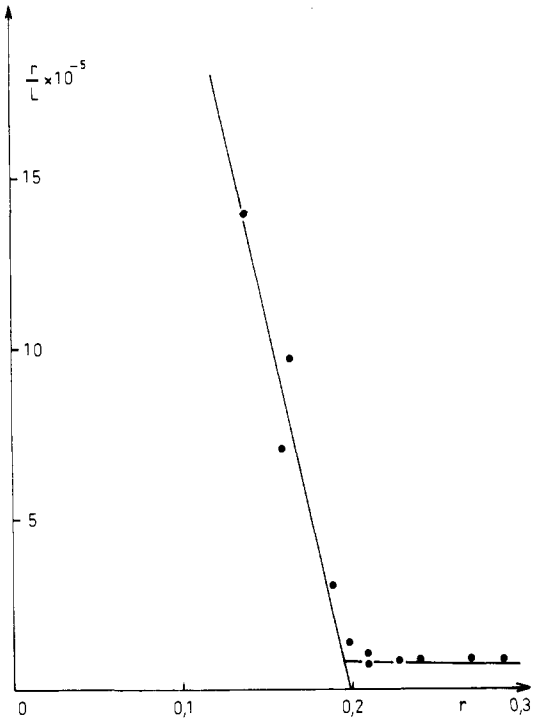

Figure 9. Titration of calf thymus DNA (8.3×10^{-6} M) in cacodylate, 10 mM, pH 7.5, by ditercalinium chloride conditions as in Figure 8.

Transient Electric Dichroism. We have investigated by TED the binding of ditercalinium to DNA together with that of an authentic intercalator from the related 6*H*-pyridocarbazole family, *N*-methylellipticine (NME).[26] The dichroism of NME and ditercalinium/DNA complexes was measured at various wavelengths. The dichroism of DNA bases has also been examined when the drug binding sites are progressively occupied (Table I). For NME, the profile of A with the wavelength of observation is similar to that of the isotropic of the isotropic absorption and, as expected, the relative dichroism A/A_i (dichroism per isotropic optical density unit) is constant. The observations with ditercalinium are dramatically different although the maximum extinction coefficients of both drug DNA/complexes are similar: at a fixed DNA concentration, the relative dichroism of NME is intense whereas that of ditercalinium is hardly detectable. Most interestingly, the dichroism of DNA bases as measured at 280 nm (where the chromophores also absorb) becomes constant after saturation of the binding sites by NME whereas it decreases steadily to zero value when ditercalinium is added. No DNA precipitation is observed. This clearly points to different modes of binding for either drug and raises the question of intercalation as being the actual interaction process, at least under the experimental conditions (low ionic strength) of the TED investigation. The absence of a dichroism of DNA-bound ditercalinium may stem from several causes. From the equation above, the dichroism can be small in the following cases: (i) The transition dipole moments are randomly distributed so that the average of $\cos^2 \Theta$ is $^1/_3$. This would indicate that the drug/DNA complex is poorly ordered as this may occur with a nonspecific binding mode of ionic origin. (ii) The angle Θ for each individual transition dipole is 55°; such a fortuitous situation can be ruled out. (iii) The orientation factor F becomes small; this could mean, in the absence of any observable precipitation, that the electric dipole moments of DNA rods decrease either by loss of DNA linear geometry for a more isotropic (spherical) one or by progressive neutralization of the electric charges on the macromolecule. In this respect, the loss of DNA dichroism where the relaxation time of the transient absorbance is unaffected (thus pointing to an unaltered viscosity and hence geometry of DNA particles) strongly suggests that ditercalinium neutralizes DNA negative charges as a result of significant ionic binding. TED experiments suffer from the inherent flaw of being compulsorily performed at low ionic strengths which would favor ionic binding over other interaction modes. However, ionic strengths as low as 1 mM have been currently used and, nevertheless, have not prevented the observation of intercalation (17–19 and NME in this study) or other groove binding processes of cationic species. Interestingly, the collapse of TED from DNA associated with ditercalinium occurs at D/B = 0.22, a value corresponding to saturation of the binding sites (See Figure 6).

Table I. Transient Electric Dichroism of DNA, DNA/NME, and DNA/Ditercalinium

NME, μM	$A_\parallel$[a] (280 nm) × 10^2	$A_\parallel$ (320 nm)	NME/DNA
0	2 (0.15)[b]	0	0
3.1	2.9	1.8 (0.16)[b]	0.09
6.2	3.4	3.5	0.18
9.3	3.8	3.7	0.26
12.5	4	3.7	0.35
156	4.2	3.8	0.44
22	4.3	3.7	0.6

ditercalinum, μM	$A_\parallel$ (280 nm) × 10^2	diter/DNA
0	2 (0.15)[b]	0
1.8	2	0.05
2.8	1.9	0.8
4.1	1.5	0.11
5.2	1.2	0.15
6.2	0.8	0.18
7.6	≃0	0.21

[a] $A_\parallel$ is the dichroism for $\alpha = 0$; TED of DNA is measured at 280 nm since at 260 nm the spectral lamp has poor energy. [b] Relative dichroism $A_\parallel/A_{iso}$ at 320 nm; at D/B = 0.09, NME is totally bound and A_{ISO} of NME at 320 nm is 0.11; TED of NME and DNA reaches constant values when NME/DNA ≃ 0.2.

T-Jump Kinetics. Acidic pH. At pH 5, when ditercalinium/DNA ratio is kept under 0.25, the temperature jump fails to induce any observable relaxation signal. This is the probable consequence of the very high affinity of the drug for DNA at this pH which prevents, at nucleotide concentrations in the micromolar range to have comparable proportions of free and bound chromophore, a necessary condition for a nonvanishing relaxation amplitude. If now the drug/DNA ratio is raised to a value larger than 0.25 (typically a ratio of 0.6 was used where precipitation of the DNA by the drug proved not to occur), a monophasic relaxation with a time constant of 50 ms is recorded. This relaxation time is similar to that observed in buffer solutions where microaggregation of the drug takes place (chloride and phosphate buffers) and, hence, is tentatively attributed to the exchange of the chromophore between the solution and the cluster.

Neutral pH. At pH 7.4 (100 mM cacodylate) and drug/base ratio = 0.6, the slow relaxation is also observed and is attributed, accordingly, to the exchange of ditercalinium with aggregates. At a drug/base ratio of 0.2, a weak relaxation (1.4 E–4 O.D. units/°C) with a time constant of 10 ± 2 ms which does not depend on the concentrations of the reacting species is recorded. The relaxation amplitude presents the same variations with the detection wavelength as already encountered earlier and

(26) Dodin, G.; Schwaller, M. A.; Aubard, J.; Paoletti, C. *Eur. J. Biochem.* 1988 *176*, 371.

typical for the complexation of the drug by the macromolecule. The kinetics at drug/base <0.25 may be understood in terms of two alternative mechanisms: (1) a process identical with that postulated above for the interaction of ditercalinium with acetate and which leads to polyionic pairs; the formal expression of the relaxation time would be like eq 1; and (2) the usual process generally proposed to account for the binding of DNA-interacting drugs and which involves the formation of an ionic complex, which does not give rise to spectral changes, followed by the migration of the bound drug to its final hydrophobic site.

In this case the general reaction scheme is

$$S + A \underset{k'_{-1}}{\overset{k_1}{\rightleftharpoons}} C_{ionic} \underset{k'_{-2}}{\overset{k_2}{\rightleftharpoons}} C_{hydrophobic}$$

Assuming that the ionic binding step is fast, as being diffusion-limited, when compared to the migration step, the expression of the observable relaxation time is

$$(\tau_2')^{-1} = k'_2 \frac{\bar{S} + \bar{A}}{[\bar{S} + \bar{A}] + K_i^{-1}} + k'_{-2} \quad (3)$$

where K_i is the equilibrium constant of the first step (ionic binding). To account for the observed lack of dependence of the relaxation time on concentrations, it must be assumed that K_i is large as compared to $[\bar{S} + \bar{A}]$.

Unfortunately, as seen from eq 1 and 3, the kinetics cannot discriminate between the ion-pairing process and the latter two-step pathway since both of them lead to formally similar expressions of the relaxation times. However, a set of indirect arguments are in favor of a significant occurrence of ion-pair formation between the polycationic drug and the polyanionic macromolecule (see discussion).

Conclusion

The experimental data that have been presented in this report have lead to the following conclusions. Ditercalinium forms strong ion pairs with most of the anions encountered in in vitro studies and present in the living cell. Pair formation may stem from the positive charges carried by the drug and from the rigid geometry of the bifunctional molecule which will favor chelation. This phenomenon is expected to occur with other polycationic "stiff" derivatives and hence should be looked for systematically in the investigation of bifunctional drug/DNA interactions. (1) At D/B ratios >0.17, bisintercalation is apparently not the binding process of ditercalinium both at pH 5 and pH 7.5. These conclusions are in agreement with the model developed by Shafer.[24,25] Monointercalation of the drug can account satisfactorily for most of the experimental results with, however, the exception of TED experiments which we consider as sound despite the reservations arising from use of low ionic strengths (see Results and Discussion). Nevertheless, the various observations lead to postulate that significant ionic bonding is involved in the interaction. These facts are (i) the marked increase of the affinity when increasing the charge of the linking chain; (ii) the absence of any dependence of the relaxation time on concentrations at pH 7.5 which stems from high ionic binding affinity (see eq 4); we observed (unpublished results) for the related 6*H*-pyridocarbazole derivatives bearing two positive charges (as diterçalinium at pH 7.5) and having the same affinity as the 7*H* compound toward DNA a marked variation of the relaxation time with reactant concentrations; (iii) the absence of TED from the bound drug and the progressive decrease of that DNA upon drug addition; (iv) the values of the relaxation times of acetate/ditercalinium and DNA/ditercalinium (pH 7.5) equilibria apparently reflect the values of their respective affinity constants (the ratios of the relaxation times and of K_{ov}/K_a being roughly 1000); this may be viewed as a consequence of the same type of association occurring in both cases, (v) if it is assumed that a monocation binds in the purely ionic mode with a constant of 1000^{-1},[23] disregarding entropy changes would lead to affinities of 10^6 and 10^9 M^{-1} for di- and tricationic species as ditercalinium is at pH 5 and pH 7.5, respectively; those values can be compared to those actually observed. Moreover, this study may provide insights into the mechanism of inhibition of the respiratory functions of the mitochondrial inner membrane. Ditercalinium is thought to act as a decoupling agent and as an inhibitor of the cytochrome oxidase. The decoupling power may stem from the fact the drug is partially protonated at a pH only slightly under neutrality and ensures proton transfer between both sides of the inner membrane. On the other hand, the inhibition of cytochrome oxidase by the parent ellipticine derivatives was understood in terms of segregation of the cardiolipid,[5] which is almost exclusively found in the mitochondrial inner membrane and constitutes the environment of the enzyme, a mechanism that accounts for the cardiotoxicity of adryamicin.[27] Since it was observed (see the introduction) that the dianionic cardiolipid does bind strongly to ditercalinium, the hepatotoxicity of the compound may be caused by this type of interaction. More generally, ditercalinium may act as a strong competitor of cations involved in the various transport processes of the cell.

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Registry No. Ditercalinium chloride, 74517-42-3; polyadenylic acid, 24937-83-5; acetic acid, 64-19-7; cacodylic acid, 75-60-5.

(27) Goormaghtigh, E.; Brasseur, R.; Ruysschaert, J. M. *Biochem. Biophys. Res. Commun.* **1982**, *104*, 314.