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PULSE FLUORIMETRY STUDY IN POLARIZED LIGHT OF DNA-ETHIDIUM BROMIDE COMPLEXES

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In previous works, a quantitative analysis of the fluorescence anisotropy decay, based on a comparison of the experimental measurements with a Monte Carlo simulation of the excitation energy migration, has been shown to provide the value of the unwinding angle of the DNA helix, induced by an ethidium bromide (E.B.) molecule intercalation. In the present work some of the characteristics of the model used in the computation are reexamined: namely the influence of the direction of the E.B. electronic moment, and the influence of the dye distribution along the DNA helix are studied. The computations are compared with experimental results obtained with new experiments performed with calf thymus and micrococcus lysodeikticus DNA-E.B. complexes. It is found that the difference in base composition of these DNA does not influence the fluorescence properties of their E.B. complexes. Our study confirms the validity of the dye distribution obtained with the single adjacent excluded site principle. Reasonable values of the unwinding angle are obtained by assuming that the transition moment direction lies along the great axis of the E.B. molecule. The value of this unwinding angle is compared with other values proposed in the literature.

1. Introduction

A number of aromatic compounds, many of them having a biological activity, can form intercalation complexes with double stranded nucleic acids, where the aromatic ring of the ligands inserts between the base planes, inducing a local unwinding of the nucleic acid helix. Consequently, when the critical value ν_c of the degree of ligand association is reached, circular DNA superhelices turns are unwound. The value of ν_c which can be determined by sedimentation velocity or buoyant density experiments, is mathematically related to the preexisting superhelix density of the DNA and to the unwinding angle δ characteristic of the ligand intercalation. Ethidium bromide (E.B.), one of these ligands, has been proposed as standard in DNA superhelix densities determination. Its unwinding angle δ has been determined by studying the complexes of E.B. with circular DNA molecules, the superhelix density of which has been obtained by controlled partial denaturation.

In previous works [1–4] we proposed an independent method of δ determination based on transient fluorescence measurements of E.B. bound to nucleic acids complexes. This method was applied to calf thymus DNA [2], poly d(A-t) of cancer pagurus [3] and to a synthetic ribonucleic acid: poly rA-rU [4].

The method rests on the following properties of the complexes:

(1) The fluorescence decay times and the affinity constants of E.B. molecules do not depend on the nature of the bases forming the binding sites.

(2) An E.B. molecule cannot be bound to a site adjacent to an occupied site, according to the model of the excluded adjacent site.

(3) The position of the phenantridinium ring was assumed to be unique and identical for all the sites.

In the present work, some of the features of the model calculation are reexamined with an improved method of analysis of the experiments. These calculations are applied to the interpretation of some new measurements performed on two DNA's having a very different base composition: calf thymus (CT) DNA and micrococcus lysodeikticus (ML) DNA.

2. Materials and methods

2.1. Biochemicals

Calf thymus DNA was kindly provided by Mme G. Aubel-Sadron. Micrococcus lysodeikticus was purchased from Miles Laboratories and was used without further purification. E.B. was a gift of Dr. Le Pecq. Two samples were studied for each DNA with P/D ratios (P = nucleotide concentration, D = dye concentration) respectively equal to 110 and 7.35. They were dissolved in a tris-HCl buffer (0.05 M) at pH 7.5 with 0.15 M NaCl.

2.2. Fluorescence measurements

The transient fluorescence has been measured with an apparatus previously described [1–6]. Excitation was provided by a light pulse vertically polarized. The two following experimental curves were determined:

$$s(t) = i_V(t) + 2\, i_H(t)$$

$$d(t) = i_V(t) - i_H(t) \qquad (1)$$

where $i_V(t)$ and $i_H(t)$ were the fluorescence components vertically and horizontally polarized, respectively. $s(t)$ and $d(t)$ were considered as convolutions of the true decay curves $S(t)$ and $D(t)$, by the apparatus response function $g(t)$:

$$s(t) = \int_0^t g(T)\, S(t - T)\, \mathrm{d}T,$$

$$d(t) = \int_0^t g(T)\, D(t - T)\, \mathrm{d}T. \qquad (2)$$

$g(t)$ was determined by means of a reference compound [7] DGDCI (diethyloxa-dicarbocyanine iodide) in ethanol solution which has a fluorescence lifetime of 1 ns.

Excitation and emission wavelengths were selected by filters (MTO 4599 and J 590 A for excitation at λ_{exc} = 521 nm, and MTO 5387 and Kodak K 55 for excitation at λ_{em} = 615 nm).

2.3. Analysis of results

The anisotropy decay $r(t)$ is defined by the following relation:

$$D(t) = r(t) \cdot S(t). \qquad (3)$$

It is assumed that $r(t)$ can be written in the following way:

$$r(t) = r_0\, r_B(t)\, r_T(t), \qquad (4)$$

where r_0 is the fundamental anisotropy, $r_B(t)$ the brownian anisotropy factor, and $r_T(t)$ the transfer anisotropy factor. $r_0\, r_B(t)$ is the anisotropy decay measured with high values of P/D.

Let us consider chromophore A excited at time zero by the incident light beam and chromophore B to which the excitation energy has been transfered at time t. If ϕ is the angle between their electronic transition moments, $r_T(t)$ is given by:

$$r_T(t) = \frac{3\langle\cos^2\phi\rangle - 1}{2}$$

where $\langle\cos^2\phi\rangle$ is the average of $\cos^2\phi$.

$r_T(t)$ is computed by a Monte-Carlo method previously described [1,2], in which one simulates the energy migration between the array of E.B. molecules bound to the same DNA molecule.

The complexes are assumed to be of the intercalation type [8]. When E.B. is intercalated it modifies locally the double helix structure of the DNA molecule. The distance between the adjacent base pairs increases by 3.4 Å and the angle between them changes by an angle δ.

$r_T(t)$ is computed with different values of δ, and $D(t)$ is determined according to expressions (3) and (4), then convoluted with the response function $g(t)$. The resulting $d(t)$ curve is compared with the experimental one, by computing the weighted mean residual WMR [9], and the deviation function.

In this calculation the following three points are reexamined:

(1) The transfer rate from a donor to an acceptor E.B. molecule is given by the Förster's formula [10]:

$$v = k_T\, \frac{\cos^2\theta}{R^6}.$$

θ is the angle between the electronic transition moments and R the distance between the two molecules. k_T is a parameter which depends on the overlap integral J, the natural lifetime τ_0 and the inverse fourth power of the DNA refractive index n_1. Several different estimations of n_1 are quoted in the literature.

The value of 1.4 [11] is probably too small; in the present work we use 1.6 and 1.75. This last value was obtained by Harrington [12] using flow birefringence measurements.

(2) In our Monte-Carlo calculation the distribution law of the E.B. molecule along the DNA helix is obtained by comparing a random number η with the "a priori" probability p that the site is occupied. Namely the site is declared occupied or not according as η is smaller or greater than p.

In our previous work [2], E.B. molecules were assumed to be distributed along the DNA molecules in such a manner as two adjacent sites could not be occupied. These conditions defined the single excluded site model [13] and were obtained by setting:

$$p = \frac{D/P}{0.5 - D/P}.$$

Other distributions can be obtained by using the more general expression:

$$p = \frac{D/P}{0.5 - K(D/P)}.$$

The cases in which no site is excluded ($K = 0$) and in which two adjacent sites are excluded ($K = 2$) will also be considered in the present work.

(3) The crystal structure of the E.B.-5 iodouracyl (3'-5') adenoside complex (14) shows that the phenantridinium ring is intercalated with the base pairs of two nucleotides in such a manner as the long axis of the dye lies between the phosphate groups of the two paired dinucleotides, while the phenyl and ethyl groups are situated in the large groove of the double helix. These structural characteristics probably occur in the E.B.-DNA complexes. One has then to consider two cases in the relative situations of two intercalated E.B. molecules. In the first case, the two molecules can be superposed by a translation of one of them along the longitudinal axis of the DNA molecule, followed by a rotation around the same axis. In the second case one must add to these operations a rotation of 180° around the short axis of the phenantridinium ring.

The angle θ between the transition moments of the two E.B. molecules is, in the first case, equal to θ_1 and, in the second case, equal to $\theta_1 - 2Y$ or $\theta_1 + 2Y$ with

$$\theta_1 = M\frac{\pi}{5} + (N+1)\delta,$$

where M and N are respectively the number of base pairs and the number of chromophores separating the two considered E.B. molecules, and where Y is the angle between the electronic transition moment and the short axis of the phenantridinium ring [11].

In our previous calculations, Y was implicitely assumed to be equal to zero or $\pi/2$. In the present work we examine other Y values. This is accounted for in our Monte-Carlo calculation, in the following way:

A number ϵ is assigned to a site occupied by a chromophore. ϵ depends on the value of the random number η which determines the occupancy of a site according to the following relations:

$\epsilon = +1$ if $\eta \leqslant p/2$,

$\epsilon = -1$ if $p/2 < \eta \leqslant p$.

Then if the donor and acceptor ϵ values are equal, θ is taken equal to θ_1. If they are different we set:

$\theta = \theta_1 + 2Y$ ($\epsilon = +1$ for donor and -1 for acceptor)

or

$\theta = \theta_1 - 2Y$ ($\epsilon = -1$ for donor and $+1$ for acceptor)

The same rule is adopted for the angle Φ between the primary excited molecule and the actually excited one. The values Y and $\pi/2 - Y$ lead to the same values of the $r_T(t)$ function since this function only depends on $\cos^2\theta$ and $\cos^2\Phi$. It is then sufficient to study the Y values comprised between 0° and 45°.

3. Results

3.1. Fluorescence decays

The fluorescence decays $S(t)$ of E.B. bound to two species of DNA (CT and ML) have been measured for several excitation and emission wavelengths (λ_{exc} and λ_{em}), and as a function of the P/D ratio.

For the two DNA's three excitation wavelengths (466 nm, 505 nm and 521 nm) and two emission wavelengths (615 nm and 651 nm) have been used to study the fluorescence decay. The P/D ratio was in both cases equal to 110.

Table 1
Values of fluorescence lifetime of E.B. bound to CT for different excitation and emission wavelengths.

λ_{em}	λ_{exc}		
	466	505	521
615	22.4 ± 0.2	22.5 ± 0.4	22.3 ± 0.7
651	–	22.3 ± 0.15	22.5

Table 2
Values of fluorescence lifetime of E.B. bound to ML for different excitation and emission wavelengths.

λ_{em}	λ_{exc}		
	466	505	521
615	22.1 ± 0.1	22.4	22.1 ± 0.4
521	–	22	22.5

All the decays can be described with a good approximation by a single exponential. The lifetime is identical for both DNA's and does not depend on λ_{exc} and λ_{em}. The results of these measurements are given in table 1 and 2. Improvement of the fits could be obtained by using a sum of two exponentials as decays. However the amplitudes and decay times of these decays are determined with a very bad accuracy. So we did not deal further with the two exponential decays.

It has been found in the previous work [1–6] that the $S(t)$ decay of E.B. bound to CT DNA was independent of P/D. This is confirmed here for the E.B.-M.L. complex as it can be seen on fig. 1. On fig. 2 the experimental decays $s(t)$ of E.B.-CT and E.B.-M.L. are compared to each other in the case of $P/D = 7.35$.

3.2. Anisotropy decays due to brownian motions

The emission anisotropy decay of E.B.-M.L. complex was measured for a sample having a P/D ratio equal to 110. The experimental data can be fitted with the following anisotropy function:

$$r_B(t) = r_0(0.5\, e^{-t/\rho} + 0.5)$$

with $r_0 = 0.32$ and $\rho = 23$ ns. This result is identical to the one obtained with the E.B.-CT complex [1,2,5,6]. We must point out however, that other expressions of $r_B(t)$ can fit well the experimental data (unpublished results). For example the following expression:

$$r_B(t) = r_0(0.35\, e^{-t/\rho} + 0.65),$$

with $r_0 = 0.32$ and $\rho = 15$ ns, gives the same convolution as the preceeding values of $r_B(t)$.

3.3. Emission anisotropy decays due to energy migration

As it can be seen in fig. 2, no appreciable differences had been found between the transient fluorescence of E.B. complexed with CT DNA or ML DNA.

These experimental curves, when analyzed with the Monte-Carlo method described above, led to the same parameters values.

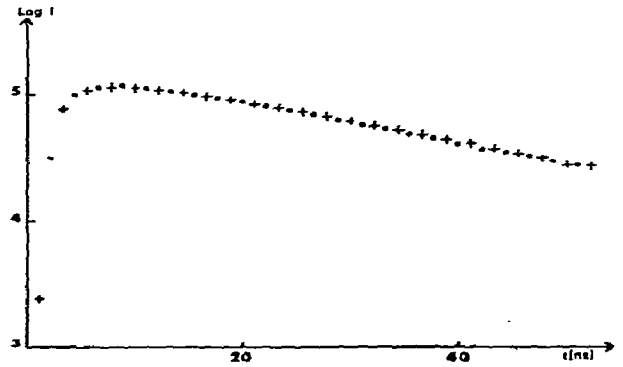

Fig. 1. Experimental $s(t)$ curves of EB-ML complex obtained for $P/D = 110$ (+++) and $P/D = 7.35$ (●●●) ($\lambda_{exc} = 521$ nm and $\lambda_{em} = 615$ nm).

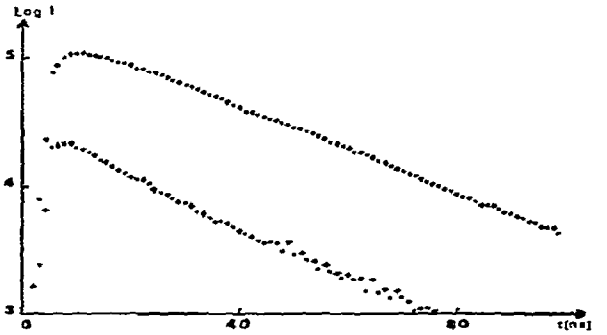

Fig. 2. $s(t)$ (upper curves) and $d(t)$ (lower curves) of EB-ML (●●●) and EB-CT (+++) complexes obtained for $P/D = 7.35$ ($\lambda_{exc} = 521$ nm and $\lambda_{em} = 515$ nm).

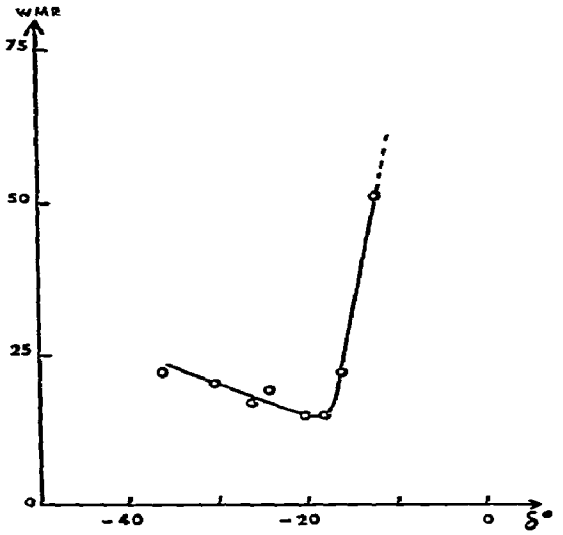

Fig. 3 EB-ML complex. WMR values versus δ computed with $n_1 = 1.6$, $J = 1.46 \times 10^{-15}$ cm^3 mole^{-1} and $P/D = 7.35$. The single excluded site model with $Y = 0°$ was used.

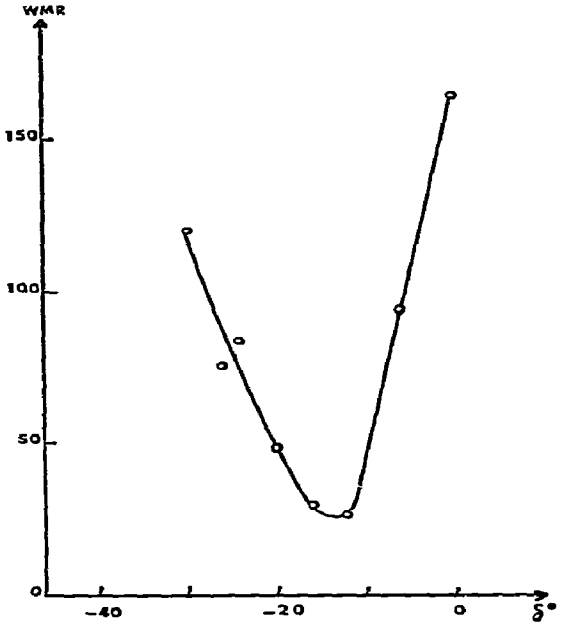

Fig. 4. EB-ML complex. WMR values versus δ computed with $n_1 = 1.75$, $J = 1.46 \times 10^{-15}$ cm^3 mole^{-1} and $P/D = 7.35$. The single excluded site model with $Y = 0°$ was used.

We first assumed that the chromophore distribution on DNA molecules follows the rule of the adjacent site exclusion and that we had $Y = 0°$. Example of curves representing WMR as a function of δ are given on figs. 3 and 4. The minimum WMR value is obtained for $\delta = -18°$ with $n_1 = 1.6$ and $\delta = -13°$ with $n_1 = 1.75$. In addition, the deviation function is randomly distributed around the time axis in the case of minimum residual values (fig. 5).

We then assumed $Y \neq 0$ and performed the Monte-Carlo calculations for two values of Y (30° and 45°). In these cases (fig. 6) the WMR curves showed a δ minimum which was respectively −4° and −36° for the two Y values.

The experimental curves were also analyzed with two other distributions. In one of them the two adjacent sites of an occupied one were excluded, and in the other, no exclusion of adjacent site was assumed. It can be seen on table 3 that for all δ values, the residual corresponding to these two last models are sensibly higher than those corresponding to the single excluded site model. Furthermore, there is no value of δ for which the deviation function oscillates randomly about the time axis (fig. 5).

4. Discussion and conclusion

Different kinds of information have been obtained from the work presented here.

The fluorescence decay time has been found independent from the emission and the excitation wavelength. It does not change with the P/D ratio nor with the base composition of DNA. Furthermore the DNA base composition does not influence the $d(t)$ curves both at low and high P/D ratios. According to our analysis, this means that the local brownian motion and the dye distribution along the DNA molecule are the same for all the sites.

Our results strongly support the single excluded site model since other models such as a two excluded sites model or a model without excluded site, cannot be fitted with our experimental results.

According to our Monte-Carlo calculation, the δ value can be determined if the transition moment direction Y is known. In these calculations, two values of Y complementary to 90° are equivalent, so are the values

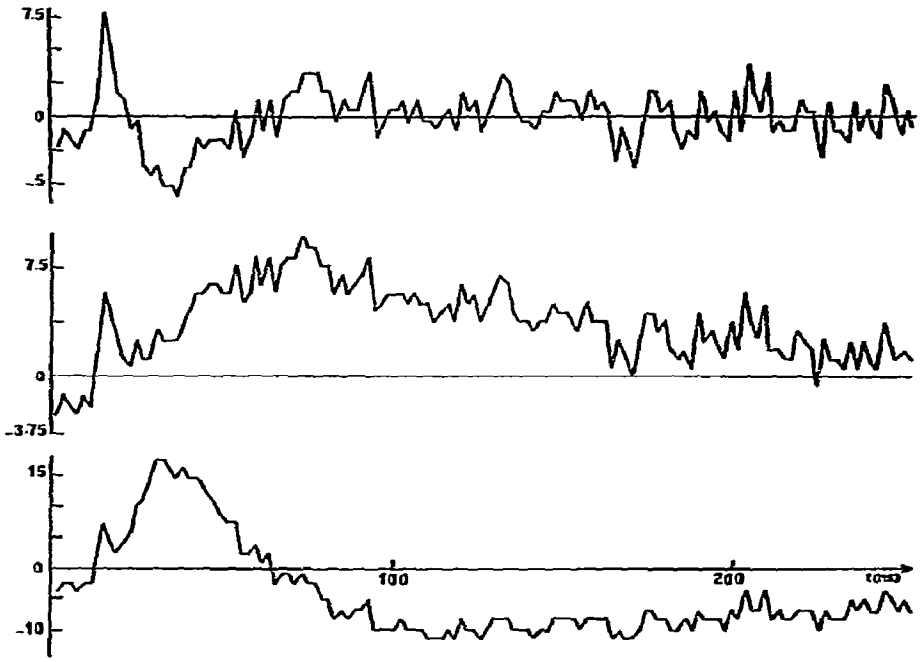

Fig. 5. EB-ML complex. Deviation functions between experimental curve $d(t)$ obtained with $P/D = 7.35$ and curves $d'(t)$ compute with $Y = 0°$, $n_1 = 1.6$ and $J = 1.46 \times 10^{-15}$ cm^3 mole^{-1} and δ given by the lower value of WMR. Upper curve corresponds to the single excluded site model. Middle curve corresponds to a two excluded sites model and lower curve to a model without excluded sites. The deviation function is defined by $[d(t) - d'(t)]/\sqrt{d(t)}$.

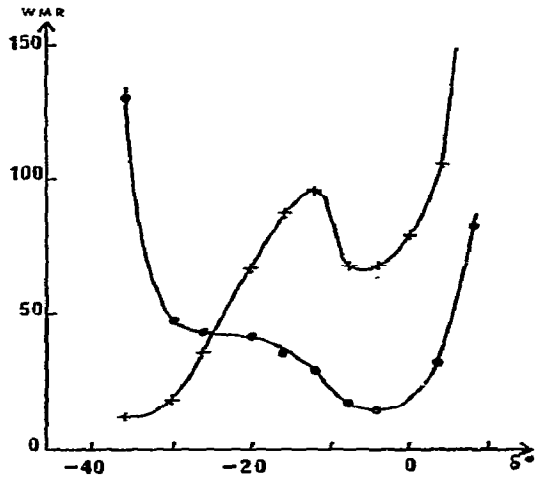

Fig. 6. EB-ML complex. WMR values versus δ for $n_1 = 1.6$, $J = 1.46 \times 10^{-15}$ cm^3 mole^{-1} and $P/D = 7.35$. The single excluded site model was used with Y values of 30° (●●●) and 45° (+++).

of 30° and 60° proposed by Houssier et al. [15] and Giacomoni and Le Bret [16] respectively, and which lead to an unwinding angle of 4°. This value is smaller than all the preceeding evaluations found in the literature and is situated outside the limits of 10° and 25° obtained by Pigram et al. [17] in their model studies. However, if Y is assumed to be 90° or 0°, more reasonable δ values are obtained.

δ depends also on the value adopted for the refractive index n_1 of the DNA molecule. With $n_1 = 1.6$ we obtained $\delta = -18°$, while with $n_1 = 1.75$ determined by Harrington, one finds $\delta = -13°$. In a previous work [2] with $n_1 = 1.4$, we obtained $\delta = -21°$.

Experimental determinations of the unwinding angle in E.B.-DNA complexes have been published in the literature. Fuller and Waring [18] first proposed $\delta = -12°$, according to model studies and X-ray diffraction patterns. Most of the determinations of δ are based on the measurements of the critical degree

Table 3
Variation of WMR with δ for a two excluded site model (2) and for a model without exclusion of sites (0).

	δ°						
	−45	−40	−26	−20	−12	0	+10
2	210	415	600	1360	810	305	300
0	–	410	–	521	–	255	–

of association ν_c which corresponds to the total unwinding of the superhelices of a circular DNA. ν_c is obtained either by sedimentation velocity of buoyant densities experiments. The superhelix density is determined by partial denaturation induced either by alcaline pH or carbodiimide. From the works of Crawford and Waring-[19] and of Vinograd et al. [20] on polyoma DNA, a value of $\delta = -15.6°$ can be obtained. Wang [21] working on PM 2 DNA found $\delta = -26°$, while with the same DNA, Pulleyblank and Morgan [22] fixed the limits of this angle between $-26°$ and $-33°$. More recently, Liu and Wang [23] determined $\delta = -24°$ by using an electron microscopy technique. On the average, these values are higher than the values obtained in our work. One of the explanations might be that the values found in the literature are obtained with circular DNA's while ours are measured with linear ones. In circular DNA, intercalation is submitted to some strains which are not present in linear DNA's.

We must acknowledge however, that the full power of our method in determination of δ will be obtained when the direction of the transition moments of the intercalated dye will be perfectly known. On the other side the results of our calculations are very sensitive to the distribution of the dye along the DNA matrix and we think that these calculations can be used for testing the distribution of the dye in other systems containing nucleic acids as, for example, nucleoproteins.

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