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Electrofluorescence of dye-tagged polynucleotides

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In general, in electric fields, molecules rotate into alignment. For polymers, tagged with fluorescent dyes, such alignment reflects in the components of polarization of the fluorescence. Results are reported of a study in which each of the three model polynucleotides poly A, poly C and poly G have each been complexed with three biologically reactive dyes. From the fast measurements of the electrofluorescent effects, some information on the type of binding geometry and the conformation of the polynucleotides can be deduced. The results would appear to indicate that the base group which characterizes the polynucleotide plays a significant role in ordering the dyes. The method could be developed to indicate base-sequence sensitivity of specific drugs for polymeric genetic material. Copyright © 1996 Elsevier Science Ltd.

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INTRODUCTION

The intense interest in the binding of carcinogens and drugs to nucleic acids[1,2] has resulted in analogous studies on simpler synthetic polymeric molecules as model systems[3,4]. In most cases the site of binding and the orientation of the carcinogen or drug on the DNA are of crucial importance because these may indicate specific mechanisms for the resultant biological activity[5–7]. In the majority of cases, these small molecular compounds have a strong interaction with light. Hence, spectroscopic techniques are well-suited to characterize the nature of the binding interactions. A number of studies have been directed at measuring the binding of chemical residues or adducts to extended polymeric molecules using optical methods[8,9]. Recent studies have embraced the analyses of the binding of drugs and carcinogens to synthetic polymeric nucleotides[10–12] composed of single base-group homopolymers or with alternating but complementary base groups such as poly(AT). The hope is that these simple structures can be used to indicate any base-sequence specificity in the binding interaction of sequence-specific drugs. There are very few techniques with which one can infer the geometry of binding directly. Among these is the measurement of the dichroism exhibited by the oriented dye–polymer array[13]. This can be used to indicate a measure of the average orientation of bound, absorbing moieties in space relative to the direction of alignment of externally constrained nucleic acid strands. A superior technique, applicable to dyes which are fluorescent upon binding, yields the data obtainable from field-induced dichroism studies plus additional information via simultaneous analysis of the emitted fluorescence from the molecular adducts. The absorption and emission analyses together greatly enhance the ability to discern the binding geometry of active fluorophores.

The absorption and re-emission of light as fluorescence at longer wavelengths are processes associated with electronic transition moments that have fixed directions within the framework of a fluorophore. Polarized incident light is optimally absorbed when the molecule is oriented with its absorption transition moment, $\underline{a}$, parallel to the polarization direction, while the emitted light is polarized predominantly parallel to $\underline{e}$, the emission transition moment[14]. When fluorescent polyelectrolytes are aligned in solution by the application of an electric field, a change may be induced in both the intensity and polarization of fluorescence that result from any ordering of the absorption and emission transition moments. Such effects have been measured on solutions of dye-tagged DNA, synthetic polymers, clay minerals and naturally fluorescent pigments[15,16]. When pulsed fields are applied, changes in the fluorescence components are transient in form. From measurements of the amplitudes of these changes at saturating electric fields, and assuming that the electric field does not change the absorption spectrum, the quantum yield, nor the motions that depolarize the fluorescence within the lifetime of the excited state, estimates of the azimuths of the transitions with respect to the alignment axes have been obtained and hence the geometry of the fluorescent dye attachment assigned. From the same experiments, rates of decay of the fluorescence changes upon removal of the field are characteristic of the time taken for the polyelectrolyte to revert to random orientational array. This has generally been taken as a measure of the size of the rotating entity[17].

In this report data are presented using this technique to study the interaction of each of the fluorescent dyes Hoechst 33258, hydroxystilbamidine isethionate (HSB), ethidium bromide (EB) and acridine orange (AO) with each of the homopolynucleotides poly A, poly C and poly G. Hoechst 33258 is a bi-benzimidazole derivative

which is an antibiotic and is used for chromosomal identification and as a marker for DNA replication studies[18]. HSB is an anti-myeloma agent of interest as a potential anti-mutagen, which binds avidly to nucleic acids and polynucleotides[19]. Ethidium bromide is a well-established chromosomal stain and DNA intercalating compound[20,21]. Acridine orange has long been known to stain nuclear material selectively within living cells[22] and to induce mutations in viruses and bacteria[23].

EXPERIMENTAL

Full details of the apparatus have been given in the literature[24,25], including the design features that nullify the inclusion of spurious scattered or polarized incident light, correct for any cell dichroic effects and hinder the influence of parasitic polarization phenomena. Procedures for sample preparation, sensitivity analysis, for data handling and analyses have all been given elsewhere[24].

Briefly, an argon-ion laser beam (*Figure 1*) of selectable wavelength in the ultraviolet or visible range, which is polarized either in the vertical or horizontal plane, is incident upon the solution, which is held in a glass cell fitted with electrodes. This incident light can be polarized either vertically or horizontally using a Fresnel-rhomb pair and supplementary polarizing prism. Electrodes in the sample cell apply a vertically directed electric field across the sample. Any fluorescent light which is emitted at 90° to the incident beam direction is analysed into its vertically and horizontally polarized components using two Glan-laser calcite polarizing prisms, one mounted on each of two detecting limbs. Suitable optical filters ensure that only fluorescent light reaches the relevant photodetector and is recorded. The efficient filtering out of any scattered laser light can be confirmed by comparing the signals from polymer solutions with and without any attached dyes. Four polarized components of the fluorescence can be measured. These are designated V_V, V_H, H_V and H_H where the capital and subscripts refer to the polarization states of the incident and analysed beams respectively. Supplementations of these components in the presence of electric fields are prefixed Δ. Any corrections required to these electric field data due to differences in the absorption of vertically and horizontally polarized light are estimated from measurements of the electrically induced dichroism of the solution[26].

The various polyribonucleotides were obtained as salts from Miles Laboratories and stored at −20°C until used. The maker's median sedimentation coefficients of these polymers in 0.05 M phosphate buffer and 0.1 M NaCl at pH 7.0 were as follows: 15.4 S for poly A, 8.0 S for poly C, and 17.6 S for poly G. The DNA sample was ex-calf thymus sodium salt of 5×10^6 daltons from Koch Light UK. The dyes were all chemically purified samples. The ethidium bromide was from BDH–Hoechst Chemicals, the acridine orange from G. T. Gurr Ltd and the sample of Hoechst 33258 was a gift from Mr Offord of Hoechst UK. Solutions were prepared by dissolving the polynucleotides in deionized and distilled water which had a specific conductivity of less than $2\,M\Omega\,cm^{-1}$. Solutions of the dye were then added to give working solutions with polynucleotide concentrations typically of 1 to $5 \times 10^{-5}\,g\,cm^{-3}$, a monomer-to-dye ratio of 250 at pH 6 ± 0.2 and 23°C. These were used within 2 h of preparation. Under these conditions of low ionic strength at this pH, DNA adopts a B helical form[1] and poly A and poly C are double stranded and possibly of B and A conformation respectively[27–29].

RESULTS

Polarization of fluorescence

The field-free polarization of fluorescence, p, which, for vertically polarized light, is defined as

$$p = \frac{V_V - V_H}{V_V + V_H} \tag{1}$$

depends upon (i) the angular difference between the absorption transition moment and the emission transition moment within the molecular framework of the dye and (ii) any movement of the dye that may occur during the interval between the light absorption and emission. A maximum p value of 0.5 occurs for those incident wavelengths and specific dyes for which the absorption and emission transition moments are parallel and which

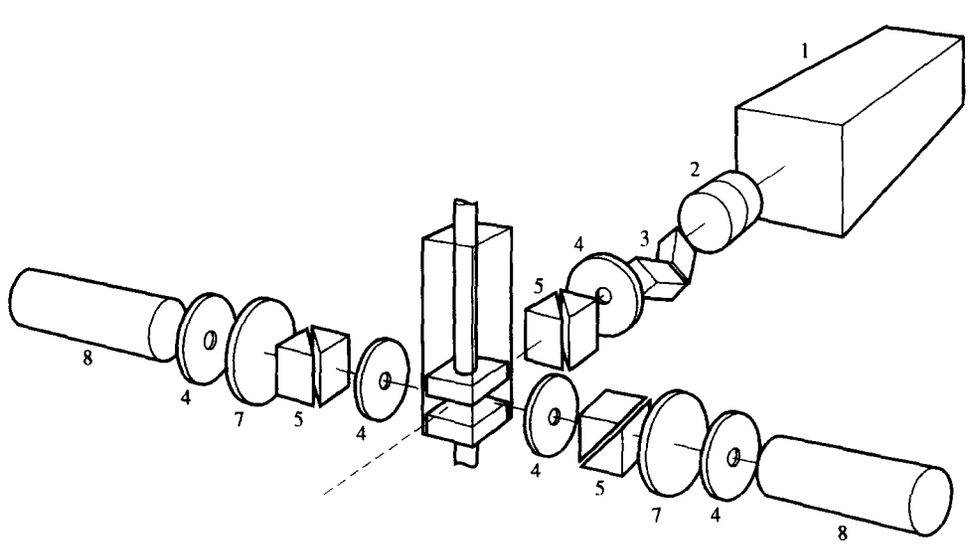

Figure 1 The apparatus. Components are: 1, laser; 2, neutral filters; 3, Fresnel rhombs; 4, apertures; 5, polarizing prisms; 6, sample cell; 7, spectral filters; 8, photodectors

are bound to a polymer or other substrate that does not significantly move within the lifetime of the excited state[30].

The p value found for dyes bound to DNA and polynucleotides when excited by ultraviolet light of wavelengths 351 nm and 364 nm (*Table 1*) fall into two categories. The first is those with low values within the range 0–0.13 and this occurs with all the AO and EB polynucleotide complexes studied here. The second is that in which p has higher values exceeding 0.30, as found for all the 33258 and HSB polynucleotide systems measured.

It is interesting to note that, in a previous study on the dyes AO and EB when bound to double-stranded DNA, for which the association is known to be via helix base-pair intercalation, high p values of 0.35 were found[31] when the fluorescence was excited with visible light (488 nm and 514 nm). For such systems the excitation wavelength was in the absorption band closest to the emission band and the absorption transition moment of the dye is essentially parallel to the emission transition moment.[32,33] This situation must also hold in the present study for the 33258 and HSB polynucleotide complexes which have high p values. In the present study, with u.v. excitation, the low p values for the AO and EB systems indicate an intrinsic angular difference betweeen the u.v. absorption transition moment and the visibile emission transition moment of the dye. We note in this respect that recent studies on acridine dyes[10] oriented in stretched polyvinyl films, indicate that the u.v. absorption transition moment (at least for the range 320–380 nm) is within the plane of the dye and directed along the long axis whereas the absorption transition in the visible spectral range is along the short axis. Because this latter axis is also the direction of the visible emission transition moment, an angular difference of 90° is indicated between the u.v. absorption and visible emission transition moments, as confirmed by our present findings. Studies on the transition moments for EB in glycerol also indicate a similar angular difference[34] between the u.v. absorption and emission moments of approximately 60°.

A further point on the EB and AO data warrants a comment. The angular differences of 90° and 60° between the relevant absorption and emission moments would be expected to give small negative values for p[30]. The observed values are small but positive, which is consistent with an additional depolarization attributable to polymer flexibility[35] and dye transition moment angles slightly modified upon binding.

The data on all systems are shown in *Table 1*. It is clear that for any particular dye, the p value is characteristic in adopting a 'low' or 'higher' value when bound to DNA or any polynucleotide. This indicates that the angular difference in absorption and emission transition moments for the bound dyes is, in all cases, similar when bound to any of these macromolecules.

Table 1 Field-free fluorescence polarization factors. Data for u.v. excitation and visible emission (> 400 nm); p is the polarization of fluorescence and f the binding enhancement factor

Dye	Polymer	p	f
Ethidium bromide	DNA	0.04	27
	Poly A	0.13	1
	Poly C	0.04	8
	Poly G	0.00	7
Acridine orange	DNA	0.08	2
	Poly A	0.08	1.5
	Poly C	0.08	0.6
	Poly G	0.12	4
Hoechst 33258	DNA	0.40	2.5
	Poly A	0.40	2.5
	Poly C	0.30	0.2
	Poly G	0.40	2
Hydroxystilbamidine	DNA	0.40	4
	Poly A	0.40	15
	Poly C	0.34	3
	Poly G	0.35	2

Intensity enhancement factor

For non-oriented media the ratio of the total fluorescence signal from the fluorophores bound to the macromolecules (I_b) can be compared with that from the same concentration of dye when free in the solvent (I_f). The ratio (f) of the two signals is the intensity enhancement factor.

$$f = \frac{(I_b)}{(I_f)} = \frac{(V_V + 2V_H)_b}{(V_V + 2V_H)_f} \quad (2)$$

Values for this factor are also given in *Table 1*. They are uncorrected for wavelength sensitivity of the photomultiplier or for any inner filter effect[14]. Both of these are considered to be insignificant at the low dye concentrations used here. High values of the ratio indicate that the fluorescence from the dye is enhanced upon binding to the polynucleotide whereas values less than unity indicate a reduction of fluorescence signal upon binding.

We note that the fluorescence from all dyes is always enhanced upon binding to DNA and similarly for all polynucleotides except poly A bound to EB and poly C bound to AO and 33258. With DNA, a ratio as high as 27 is indicated and this is consistent with an intercalative mode of binding to a double-helical structure[21]. Enhancement is also strong for HSB on poly A where a ratio of 15 occurs. Among the systems studied, reduction of the fluorescence signal only occurs for poly C when bound with the dyes 33258 and AO. A further noteworthy point for the HSB fluorescence is that the appearance depends on the polynucleotide to which it is bound. When free in water it has a dull red emission, which becomes bright red when bound to DNA and fluoresces white on poly A, bright pink on poly C and yellow on poly G.

Field induced changes

Regular transient responses were observed (*Figure 2*), for all dye–polynucleotide complexes consistent with effects which originate as a result of some degree of uniform orientation of the dyes on each of the polymer matrices. Random dye orientation on the polymers would have given no transient responses upon polymer alignment. Relative amplitudes and relaxation times for the electrically induced changes in the polarized components of the fluorescence for all dyes and polynucleotides are listed in *Table 2*. Changes in the relative component amplitudes can be interpreted to indicate the average binding geometry of the planar dyes[24] relative to the nucleotide axis (see below), which is the orientation axis.

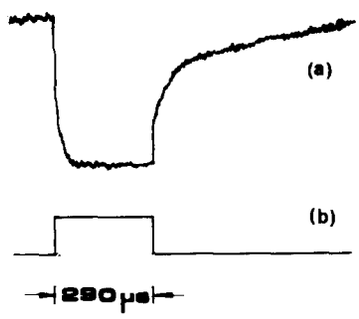

Figure 2 Typical transient electro-fluorescent response. Trace for the change in the V_V component for a sample of poly C–ethidium bromide complex with u.v. light excitation. The applied field pulse (lower trace) of 15 kV cm^{-1} amplitude and 290 μs duration, runs in time from left to right. The transient response (upper trace) displays the finite time to return to ambient orientation and shows the typical noise level of the responses

DISCUSSION

There are four general features that aid the data analysis. The first is the signs of the changes in the relative polarization components of the fluorescence. These indicate the directional characteristics of the planar dyes relative to the major polymer alignment axis. An example of the reasoning is given in the section discussing data for the DNA–EB complex. Fuller enumeration of the relative directions of the absorption and emission moment directions is accessible from the magnitudes of the component changes[17]. The transition moments for the absorption and emission of visible light generally lie in the plane of the dye molecules[36]. This is assumed, to a first approximation, also to be the case for u.v. excitation for the dyes studied here. Secondly, the relaxation time of the post-field decay of the fluorescence polarized components is indicative of the rotary relaxation of the polymers and for single-stranded polynucleotides is expected to be faster than 50 μs. Values in excess of this indicate enhanced molecular sizes such as those encountered with double-stranded structures.

The third feature is that high values of p indicate structures in which the absorption and emission moments are close to parallel, a feature which can be confirmed for the averaged moments within the complex structure by the fluorescence cross-term changes, i.e.

$$\left(\frac{\Delta H_V}{H_V}\right) \quad \text{and} \quad \left(\frac{\Delta V_H}{V_H}\right)$$

being approximately equal[15].

Finally, differences between these cross terms, especially when of opposite sign, readily indicate a wide angular separation between the absorption and emission moment directions within the macromolecular complex.

Ethidium bromide complexes

For EB and DNA, the nature of the general dye ordering can be deduced from the components with the use of vertically polarized light (*Table 2*). An applied electric pulse gives rise to a vertical electric field which orients the DNA helices towards the vertical. This is accompanied by a decrease in both the vertically and horizontally polarized emissive components as both ΔV_V and ΔV_H are negative. The total fluorescence intensity in the electric field is given by the sum $[(V_V + \Delta V_V) + 2(V_H + \Delta V_H)]$ and is, overall, a reduction. Hence, the amount of light absorbed is reduced upon alignment and the absorption transition moment is associated predominantly with the horizontal plane. The bound fluorescent dyes must therefore align away from the direction of the electric vector of the incident light and lie predominantly perpendicular to the orientation axis of the DNA. From a comparison of the intensity of fluorescence polarized in the vertical direction $(V_V + \Delta V_V)$ with that polarized in the horizontal $2(V_H + \Delta V_H)$ plane, the emission moment direction can be estimated. Because ΔV_V is more negative than $2\Delta V_H$, the emission is more strongly polarized in the horizontal plane than in the vertical and the emission moment must also be predominantly perpendicular to the DNA axis.

Table 2 Normalized fluorescence polarization component changes. Data for u.v. excitation and visible emitted light with high fields in the saturation region

Dye	Polymer	$\frac{\Delta V_V}{V_V}$	$\frac{\Delta V_H}{V_H}$	$\frac{\Delta H_V}{H_V}$	$\frac{\Delta H_H}{H_H}$	τ (μs)
Ethidium bromide	DNA	−0.68	−0.31	−0.51	+0.66	300
	Poly A	−0.42	−0.28	−0.28	+0.19	100
	Poly C	−0.63	−0.33	−0.34	+0.45	400
	Poly G	−0.42	−0.17	−0.30	+0.46	20–50
Hoechst 33258	DNA	+0.40	+0.25	+0.32	−0.38	300
	Poly A	−0.66	−0.35	−0.35	+0.53	120
	Poly C	−0.27	−0.04	−0.04	+0.24	–
	Poly G	−0.63	−0.37	−0.39	+0.45	20–50
Hydroxystilbamidine	DNA	+0.12	+0.30	+0.28	−0.22	–
	Poly A	−0.70	−0.38	−0.38	+0.53	120
	Poly C	+0.20	−0.00	−0.04	−0.16	–
	Poly G	−0.33	−0.27	−0.27	+0.37	–
Acridine orange	DNA	−0.51	+0.29	−0.52	+0.23	350
	Poly A	−0.45	+0.21	−0.49	+0.22	130
	Poly C	−0.23	+0.14	−0.18	+0.13	–
	Poly G	−0.50	+0.25	−0.46	+0.22	20–50

Since, for these wavelengths, both the absorption and emission transition moments lie in the plane of the dye and at 60° to each other[34], the data are consistent with an intercalation of the plane of the ethidium dye between the base pairs of the DNA[37–39]. A similar conclusion can be obtained from consideration of the relative component changes in fluorescence when horizontally polarized incident light is used. In the following text, similar reasoning can be used to indicate when dye molecules bind predominantly parallel or perpendicular to the polymer axis (*Figure 3*). An exact theory exists[17] for the enumeration of the inclination angles but only for rigid rod-like molecules which these systems are certainly not.

With the EB–poly C complex effects are obtained which are similar in sign and magnitude to those found for DNA. A high degree of uniform alignment of the dyes on this polymer are indicated with the absorption and emission transition moments perpendicular to the polymer axes. Hence, the dye planes are essentially perpendicular to this axis. There is, however, one difference between the data found for poly C and DNA in that the cross terms $\Delta V_H/V_H$ and $\Delta H_V/H_V$ are similar for poly C but different with DNA complex.

Theoretical calculations[15] indicate that non-equality of the cross terms occurs when the absorption and emission moments have different azimuths with respect to the alignment axes. Differences in the details of the apparent intercalative binding are thus indicated.

From *Table 2* it can be seen that equal cross terms occur for systems with both low (0–0.13) and high (0.30–0.45) values of p whereas different cross terms occur only for systems with low p values. Data from the electro-fluorescence cross terms and the field–field polarization parameter p are in concord on this point. The data for the intercalation of EB into poly C where the polarization is low but where experimental polarization cross terms are equal, may be interpreted as follows. The similar cross terms indicate similar azimuths for the average $\underline{a}$ and $\underline{e}$ per molecule with respect to the applied electric field direction. At saturating orientation the transition moments of the dyes must have the same average azimuths on the polymer axis. Because $\underline{a}$ and $\underline{e}$ are not parallel, and the plane of the dyes is not truly perpendicular to the polymer long axis (for component ratios would then be closer to unity), the most probable explanation for apparently similar azimuths for $\underline{a}$ and $\underline{e}$ would be that the dyes bind along each polymer chain with a range of azimuths for both $\underline{a}$ and $\underline{e}$, but so that the average azimuths resolve to be the same. An illustrative example of this possibility is *Figure 4*, which shows the projection on a perpendicular plane of neighbouring dyes oriented along the chain. For every azimuth, $\underline{a}$, there is a similar azimuth for $\underline{e}$, although arising from a different moiety along the chain. The number of dyes bound to a polymer with the absorption moment $\underline{a}$ in a given range of azimuth angles is the same as the number with the emission moment, $\underline{e}$, in that range.

The data for EB bound to DNA with low polarization and different cross terms, indicates different azimuths for $\underline{a}$ and $\underline{e}$. A more specific orientation for the dye plane is thus indicated. The dyes must bind to the polymer so that the average values of the azimuth for $\underline{a}$ and $\underline{e}$ differ. We note here that this is inconsistent with the dye plane being exactly perpendicular to the orientation axis but is consistent with a specific tilted dye plane, as illustrated in *Figure 5*. It is apparent in this binding model that although the dyes may be rotated and translated along the polymer axis, the azimuths of $\underline{a}$ and $\underline{e}$ remain constant at each site. It is therefore likely that the dyes adopt a fixed orientation to the base groups at each binding site (unlike type 1 binding). This is referred to as 'type 2' intercalation.

This point of structural finesse between type 1 and 2 intercalative binding can only be discerned from electrofluorescence data. Dichroism only indicates the influences of the single $\underline{a}$ vector. The EB–poly A complex has low polarization, but similar cross terms and relative components of similar sign to those of an intercalator but

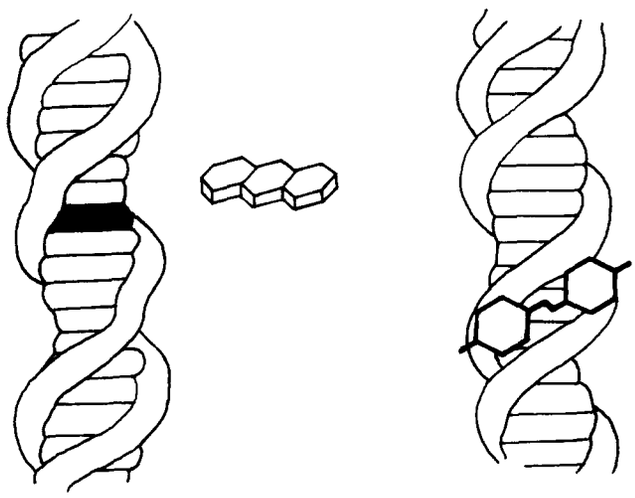

Figure 3 Diagrammatic representation of dye-binding to DNA. Conventional perpendicular intercalation (left) and minor groove binding (right) are shown

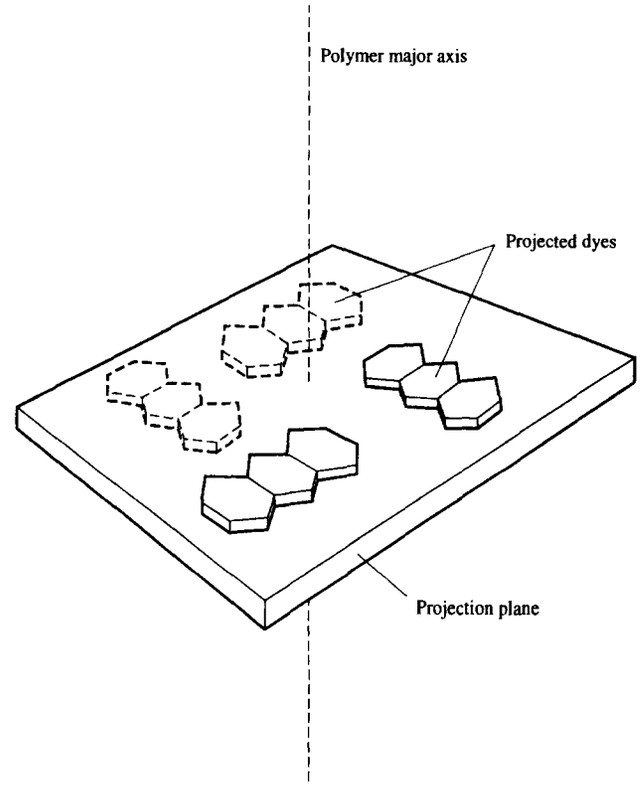

Figure 4 'Type 1' intercalation, in which the dye molecules along the polymer chain project on a plane perpendicular to the backbone to negate any radially resultant binding direction

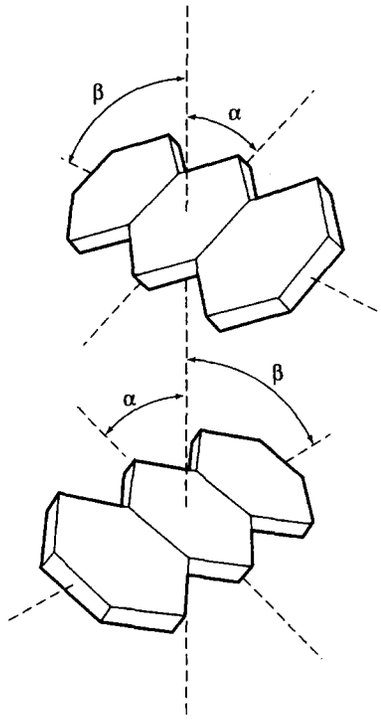

Figure 5 'Type 2' intercalation. The dye planes are tilted as they bind essentially perpendicular along the polymer major axes

with lower magnitudes. A type of binding similar to poly C with EB is indicated, except that there is a larger range of limiting azimuths. Relaxation times indicate a double-stranded structure. This dye is less well ordered within the poly A structure than the other dyes mentioned below. The fluorescence intensity is not enhanced, and this is exceptional for an intercalating dye.

For the EB–poly G complex, one sees relative component changes similar to ethidium bromide bound to DNA in that the cross terms differ from each other while the magnitudes of the relative components are smaller than for DNA. This may be due to an absorption transition with smaller azimuth or to some degree of random binding. In addition, the relaxation times are low compared with DNA, indicating a single-stranded structure. A type 2 form of intercalative binding is indicated but on a single-stranded structure. This structure must, nevertheless, have a highly ordered arrangement of base groups to produce such uniform dye orientation compared with the double-stranded structures encountered with poly A and poly C.

Acridine orange complexes

Acridine orange bound to DNA is a well-known intercalator into the double-helical structure. Relative components of fluorescence have been measured using visible light excitation, where the cross terms were the same and the polarization parameter, p, was in the high range[16].

The data of *Table 2* are also consistent with a perpendicular binding of the dye plane to the polymer axis. The relatively large differences in the cross terms, which are of different sign, indicate a highly ordered type 2 mode of binding. The cross term difference is more marked with acridine orange than with ethidium bromide. This does not necessarily indicate an increased tilting of the dye plane for acridine orange compared with ethidium bromide since the intrinsic angular discrepancy between the two transition moment directions is known to be approximately 90° for acridine orange and closer to 60° for ethidium bromide. Hence, one would expect acridine orange to give a greater difference in azimuth than ethidium bromide for the same plane tilt.

With AO–poly A the relative components show a similar behaviour to the acridine orange–DNA system in both magnitudes and signs; the long relaxation times indicate dyes bound to double-stranded material. A highly specific binding occurs which is consistent with type 2 association. The data are also compatible with tilting dyes, with the dye plane inclination similar to that found for acridine orange bound to DNA.

Comparison of the intensity of fluorescence obtained with visible excitation[16] and the u.v. excitation of this study, indicates a greater azimuth for the visible absorption transition. Hence, the long axis of the AO is less inclined than the transverse axis to the polynucleotide backbone.

For the AO–poly C complex, the relative fluorescence components have lower magnitude than for acridine orange bound to DNA. The intensity of fluorescence is therefore greater in the field, although the signs of the changes are similar and a large difference in the cross components exists. A specific binding is indicated but with a less perpendicular arrangement for the absorption transition moment. A smaller azimuth of the dye long axis may be indicated. This is consistent with smaller residual dichroism found for this system which indicates a higher tilting of the bases for poly C than for poly A[29]. Alternatively any randomly bound dye molecules could, if their fluorescence is not quenched, reduce the magnitudes of the relative components disproportionately. This second possibility for dye binding would be more influential if the fluorescence was not quenched to the same extent as that experienced for any ordered bound dye. Because of the low fluorescence yield with this system, the signals were too noisy to allow accurate assessments of the relaxation times.

Results obtained for the AO–poly G system show a remarkable similarity to those found for the acridine orange–DNA complex, so that a similar geometry of binding is assumed. The electro-fluorescence data indicate that the acridine planes bind with a similar tilt to the polynucleotide as that found when bound to double-helical DNA.

Hoechst 33258 complexes

Data for the 33258 and DNA system are entirely consistent with those reported[40] in previous studies in which the dyes bind to the double helix with their in-plane transition moments oriented somewhat parallel to the helix long axis.

In the 33258–poly A complex the binding is similar to the acridine orange–DNA (and acridine orange–poly A) systems when visible light was used to excite the fluorescence. The data are consistent with the situation where a is parallel to e. The cross terms are consistent with the high p values found here. The relaxation time is long and indicates a double-stranded structure for poly A.

Clearly, the groove-binding mode favoured for Hoechst 33258 to DNA does not occur in double-stranded poly A, presumably because any groove structure is less well defined or not sterically suitable. The fluorescence enhancement is similar. However, the

intercalation orientation achieved for typical planar dyes with parallel transitions (such as for AO–DNA with visible excitation) occurs for Hoechst 33258 bound to poly A. The similarity in magnitudes of components suggests similar azimuths for both a and e and a similar secondary structure for these bases within poly A as for DNA.

The 33258–poly C system proved to be difficult to measure as the fluorescence was always quenched. Nevertheless, from the small but finite changes in the fluorescence components, intercalative binding is again indicated. Although the low magnitude suggests a high degree of tilt, together with some background random binding, relaxation times were too imprecise to measure. The ordering of Hoechst 33258 on this polymer is very indiscrete. We note that low ratios of the enhancement factor, f, correlate with relatively small magnitudes for fluorescent component changes. It is clear that the yield of fluorescence is related to the ordering of the dyes.

Measurements on the 33258 and poly G gave relative fluorescence component changes that were similar in sign and magnitudes to intercalated dye–DNA complexes with high p and equal cross terms. In these respects the data are the same for poly A binding to 33258. The relaxation time from the poly G complex is short (50 μs or less), indicating that the polymer molecules are single stranded. A highly ordered, single-stranded structure is again indicated, which differs from the DNA complexes with this dye. The groove-binding mode seen with DNA is thus not present for these polynucleotides.

Hydroxystilbamidine complexes

As mentioned earlier, the HSB–poly A system showed a fluorescence enhancement of 15 times, which was much greater than that seen with DNA (*Table 1*). Previous studies have indicated that this dye does not intercalate and is therefore a probable groove binder to native DNA[15,41,42]. The magnitude and size of the relative components for HSB–poly A are similar to those seen with visible excitation for acridine orange bound to DNA[31], indicating perpendicular binding with similar polarization values. The complex has a long relaxation time. Hence, the data indicate binding of HSB to double-stranded poly A with a very large increase in fluorescence. The dye does not appear to bind in the grooves of poly A. The absorption and emission transition azimuths of HSB on poly A are very similar to those for acridine orange bound to poly A, and to DNA, and EB bound to DNA using visible radiation.

Polarization values for the HSB–poly C complex indicate parallel absorption and emission moment transitions. The electro-fluorescence component changes are small but similar in sign to those for a groove-binding dye interaction. The fluorescence is pink. With DNA the colour is bright red and thus a potential chemical binding indicator is identified. In addition, the dye binds with the absorption and emission transitions neither predominantly perpendicular nor parallel to the polymer backbone. This indicates a non-intercalative mode of binding and may be consistent with the dye molecules lodging in the helical groove of a double-stranded poly C conformation.

For the HSB–poly G system, the fluorescence is very yellow in appearance and in the electric field, the signs of the components are again consistent with intercalative or perpendicular dye-plane binding. The small relaxation time reveals that the dyes bind to single poly G strands in solution. The magnitude of the effects are small compared with those seen when the dyes intercalate the DNA and a low degree of uniform dye ordering on single-stranded polymer is indicated.

CONCLUSIONS

The following general conclusions are drawn for the dye–polynucleotide interactions:

- association of the dye molecules with their planes essentially perpendicular to the polynucleotide backbone, which is indicative of intercalative binding, appears to be a favoured binding mode for dyes to polynucleotides with a single base type, and occurs with both double- and single-stranded polymers
- for each of the polynucleotides studied dye intercalation can occur with the in-planes axes of the dye, uniformly tilted at each site of binding. Tilting in poly A and poly G is similar to that in DNA but may be more inclined in poly C.
- whereas all homopolynucleotides studied have an intercalative mode of binding for Hoechst 33258, this fluorophore binds quite differently to native DNA, where the dye planes relate more closely to the nucleic acid long axis
- for EB, an intercalative binding type which involves random rather than uniform tilting at each binding site in double-stranded poly A and poly C is indicated
- poly G is exceptional in that it has a low relaxation time indicative of a single-stranded structure; even so, the dye ordering, which includes the unformly tilted type, is similar to that found for DNA
- the poly C structure produced the greatest variability in dye ordering, enhancement factor, colour change and relaxation time. This polynucleotide exhibits the greatest sensitivity to the nature of the dye.

It is also concluded that the role played by the base groups in the conformation and the binding properties of mono-polynucleotides in the presence of interactive and biochemically significant dyes, is of importance. The electrofluorescence method is able, with relative speed and sensitivity, to indicate these variabilities. Studies can be extended to polynucleotides with alternating and complementary base groups to probe for any correlations between the effectiveness of certain drugs and any base-sequence specificity in their binding characteristics to genetic material.

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