of the PSB system and find, in agreement with our results, that isomerization should take place efficiently out of the lowest $^1\pi\pi^*$ state.

On the basis of a lack of any O_2 effect on the isomerization yield of retinal, Ottolenghi et al.[20] ruled out a thermalized triplet state as a precursor to the isomerized species. Our mechanism implicates the thermalized triplet in isomerization and we explain Ottolenghi's observation by the alternative assumption that O_2 quenches the thermalized triplet without substantially effecting its branching ratio into the various ground-state isomers. Our explanation would apply whether the triplet existed in one common, presumably twisted, conformation or as a mixture of all isomers. Ottolenghi further observed that triplet-sensitized isomerization occurred from the 11-cis isomer but not from *all-trans*-retinal. This interesting result he took as further evidence that the thermalized triplet is not involved in isomerization. He explained the observation by assuming that isomerization from a nonrelaxed triplet can efficiently compete with thermalization in the case of 11-*cis*-retinal but not of all-trans. We propose that in the sensitization studies, insufficient energy is available for the all-trans triplet to overcome the potential barrier and reach the twisted triplet configuration.

Finally, we would like to point out that our observation of a single isomerization lifetime faster than 10 ns is consistent with the observation of Busch et al. that prelumirhodopsin is formed from rhodopsin in a matter of picoseconds.[11] This gives us more confidence that a protonated retinylidene Schiff base is a good model system for rhodopsin. The vastly different behaviors of retinal and its Schiff base point out the danger in using these compounds as rhodopsin model systems.

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Structural Implications of the Electronic Spectra of Quinacrine–Deoxyribonucleic Acid Complexes in the Ultraviolet Region (250–300 nm)[1]

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Abstract: Ultraviolet difference absorption spectra have been measured for quinacrine-polynucleotide complexes. The difference absorption spectra of quinacrine-native calf thymus DNA and quinacrine-poly(dA-dT)·poly(dA-dT) complexes include two bands at 270 and 290 nm, whereas in the spectrum of quinacrine itself only one band at 280 nm is observed. In the difference spectrum of quinacrine-poly(dG)·poly(dC) complexes, a broadened band is observed in the 280-nm region. The effect of the phosphate/dye (P/D) ratio on the observed absorption band splitting indicates that the splitting resulted from highly localized nearest neighbor interactions of the dye and nucleic acid bases. It does not originate from dye-dye interactions. Observation of an isosbestic point in these studies indicates that the splitting was associated with only one type of binding, the strong "primary" binding process. The absorption band splitting is attributed to the interaction between exciton states of the complex and requires the close proximity between the dye and nucleic acid bases. We conclude that quinacrine is bound closer (intercalated) to A-T base pairs than to G-C base pairs in the polynucleotides. Fluorescence quenching experiments with iodide ions confirm these conclusions.

Recently several workers[3-8] have studied the complexes formed between the antimalarial drug and chromosomal staining agent quinacrine (QAC) and nucleic acids. This interest has been stimulated by the potential use of complexes formed between cationic acridine dyes and nucleic acids as regioselective labels in the staining of chromosomal DNA in

eukaryotes[9-13] and the wide variety of biological effects in which the interaction between the dye and nucleic acid have been implicated.[14-16] Relationships based on the variation of QAC fluorescence, which depends on the nucleic acids in the complex, have been developed. However, the structure of the complex has not been unambiguously determined.

The interaction of molecules in a complex will influence their spectral properties. Dispersion interactions[17a] result from the interaction of the permanent moments of the molecules and second-order perturbation of their transition moments. Only minor changes in shape and position of the absorption spectrum occur, providing general (nonspecific) information on the molecules' local environment. Spectral solvent shifts are examples of the effects of dispersion interactions.

Excitation exchange interactions[17b] between neighboring molecules arise from the first-order perturbation of the transition moments of the interacting molecules, forming exciton states, which manifest themselves in the observation of Davydov splitting in the absorption spectrum. The magnitude of this type of interaction is strongly dependent on the energy difference, orientation, and nature of the interacting transition moments. Potentially this type of interaction could provide more specific information on the molecular environment. Because of the r^{-3} dependence of exciton interaction, one would expect to observe these perturbations in the spectra of dye–polynucleotide complexes when the dye is particularly close to the bases of the polynucleotide in the complex.

Earlier we reported[3] the observation of splitting in the ultraviolet difference absorption spectra of QAC–polynucleotide complexes. These results indicated that splitting depended on the particular polynucleotide in the complex and could be related to the distance of approach between the base pairs and the dye. We suggested that splitting in the ultraviolet difference absorption spectra may originate from exciton interactions between the excited states of dye and polynucleotide. Our preliminary results, however, did not conclusively characterize the interactions which resulted in the spectral modifications. Alternative explanations, though less satisfactory, had not been completely eliminated.

We have conducted further experiments which are reported in this paper which clarify the origin of the splitting. These results indicate that studies of the ultraviolet difference absorption spectra can be useful in describing the nature of the interactions in dye–nucleic acid complexes and can be a diagnostic for helix–coil transitions.

Experimental Section

All solutions were buffered at pH 7 with 0.01 M sodium cacodylate unless otherwise noted. Fresh stock solutions of QAC were prepared each day and solutions of QAC and polynucleotide were used within 1 h of preparation. Calf thymus DNA was purchased from Worthington Biochemicals, Freehold, N.J. The synthetic polynucleotides poly(dA-dT)·poly(dA-dT) and poly(dG)·poly(dC) were purchased from either Miles Laboratories, Kankakee, Ill., or P-L Biochemicals, Milwaukee, Wis. The polynucleotides were dialyzed against cacodylate buffer for 24 h prior to use. The degree of duplex structure of the polymers was estimated by thermal denaturation analysis using a Gilford 2427 thermoprogrammer. The hyperchromicities of the calf thymus DNA, poly(dA-dT)·poly(dA-dT), were comparable to the commercial values, while the poly(dG)·poly(dC) hyperchromicity was 15% lower than the commercial specifications. Quinacrine dihydrochloride was purchased from Sigma Chemicals, St. Louis, Mo., and was purified by recrystallization.

To measure the influence of a singlet quenching agent on the fluorescence of either free QAC or complexed QAC, fluorescence decay rates and changes in the fluorescence intensity were studied. In the fluorescence intensity experiments, iodide ions (NaI) were used as an appropriate quencher with the ionic strength of the system being kept constant at 0.1 M by the addition of appropriate amounts of NaCl such that $[Cl^-] + [I^-] = 0.1$ M. Steady-state fluorescence intensities were measured with a Farrand MK-1 spectrophotofluorimeter with excitation at 365 nm (bandwidth 5 nm), where the molar extinction coefficients of the bound and free QAC are similar, and emission observation at 490 nm (bandwidth 10 nm; QAC emission maximum).

Polarization excitation spectra were measured on an NRC built spectrophotofluorimeter. The excitation source consisted of a 1000-W xenon arc lamp in a Schoeffel lamp housing and two Jarell Ash (Model 82-410), 0.25-m monochromators coupled together. Fluctuations in lamp intensity were corrected by placing a beam splitter in the excitation beam with the reflected beam being detected by an ethylene glycol solution of rhodamine B as the quantum counter.[18a] Plane polarized light was obtained by the use of a Glan calcite polarizer. The emission having passed first through a polarizing film and then a 3-70 Corning filter was detected at right angles to the excitation beam with an EMI 6256 B photomultiplier tube. The polarized excitation spectrum was then determined from the formula[18b]

$$\rho = (I_{VV} - TI_{VH})/(I_{VV} + TI_{VH}) \quad (1)$$

where $T = I_{HV}/I_{HH}$ is the correction factor which was determined as described by Azumi and McGlynn.[19] Appropriate corrections were made for blanks. The excitation band width was 1.7 nm. Fluorescence lifetimes were measured on a TRW fluorescence lifetime apparatus adapted to use time-averaging techniques[20] to improve decay time results. The decay times measured were accurate to within ±1 ns which is sufficient accuracy for the purposes of these experiments.

Difference absorption spectra measured on a Cary 15 spectrophotometer (dispersion 20 Å/mm) were used for the detailed study of the 250–350-nm spectral region of the polynucleotide–dye systems. The following standard procedure was used. A solution of the nucleic acid to be examined was divided into two equal parts in 1-cm cells at 23 °C. One cell was put into the sample compartment and the other into the reference compartment of the Cary 15 spectrophotometer and the zero absorption line was calibrated. Then a small volume of the dye in the buffer solution was added to the sample cell and equal volume of the buffer solution was added to the reference cell. Both cell solutions were smoothly stirred, and after 10 min the difference absorption spectrum was measured. Identical maxima were observed for reference absorbancies of 0.6 (slits, 1 mm) and 1.85 (slits, 2.5 mm).

Results and Discussion

Difference Absorption Spectra. The absorption spectrum of a buffered solution (0.01 M cacodylate, pH 7) of QAC is shown in Figure 1. The spectrum is characterized by three broad bands centered at 22 470 (445 nm, $f = 0.17$), 29 940 (334 nm, $f = 0.07$), and 35 710 cm^{-1} (280 nm, $f = 1.08$). The polarized fluorescence excitation spectrum of QAC in glycerol (Figure 2) clearly shows that these bands correspond to three distinct electronic transitions. The absorption bands are, therefore, assigned as being the $S_0 \rightarrow S_1$, $S_0 \rightarrow S_2$, and $S_0 \rightarrow S_3$ electronic transitions in QAC, respectively. The polarized fluorescence excitation spectrum shows that the $S_0 \rightarrow S_2$ and $S_0 \rightarrow S_3$ transitions are negatively polarized with respect to the $S_0 \rightarrow S_1$ transition. These results are in close agreement with those of Zanker and Wittwer[21] who studied the polarized excitation spectra of several 9-aminoacridine derivatives.

Consideration of the absorption spectrum of DNA (Figure 3) indicates that excitation exchange interaction would only occur between the S_1 excited state ($S_0 \rightarrow S_1$ at 38 460 cm^{-1} (260 nm, $f = 0.10$)) of DNA and the S_3 state of QAC. The ultraviolet absorption spectrum of the QAC–DNA complex (Figure 3) was clearly not additive, i.e., the sum of the individual absorbancies of QAC and DNA alone. Therefore some spectral perturbations of the QAC and/or DNA transitions were evident. However, in order to ensure that only strong "primary" binding was occurring,

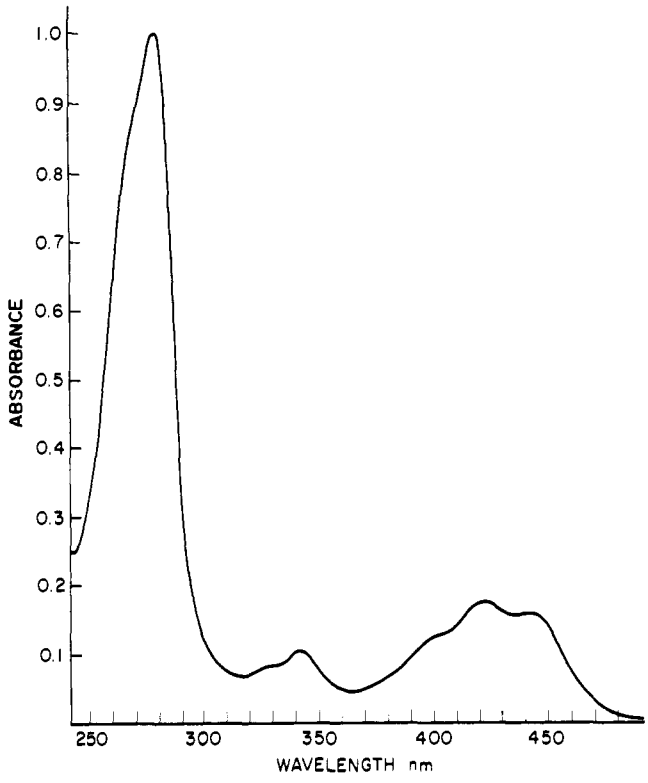

Figure 1. Absorption spectrum of quinacrine (1.89×10^{-5} M) in aqueous cacodylate buffer (0.01 M, pH 7).

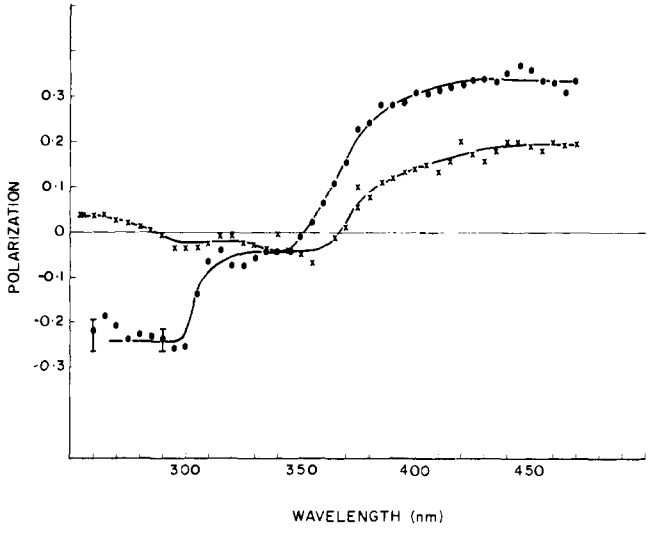

Figure 2. Polarized fluorescence excitation spectra: (●) quinacrine (3.1×10^{-7} M) in glycerol; (X) quinacrine (3.1×10^{-7} M)-poly(dA-dT)·poly(dA-dT) (6.5×10^{-6} M), P/D = 21, in cacodylate buffer (0.01 M, pH 7).

we worked at phosphate to dye (P/D) ratios of usually 20:1, in which case the strong 260-nm absorption band in the spectrum of the polynucleotide, which is largely unperturbed by the bound dye, masked any perturbation of the dye transition in the same spectral region. The difference absorption spectra (Figure 4) were therefore recorded for QAC and three different polynucleotides: native calf thymus DNA, poly(dA-dT)·poly(dA-dT), and poly(dG)·poly(dC).

In the difference spectrum of the QAC-DNA complex (Figure 4a), again several bands are observed. The bands now observed at 455 and 350 nm, corresponding to the $S_0 \rightarrow S_1$ and $S_0 \rightarrow S_2$ transitions, are shifted relative to the

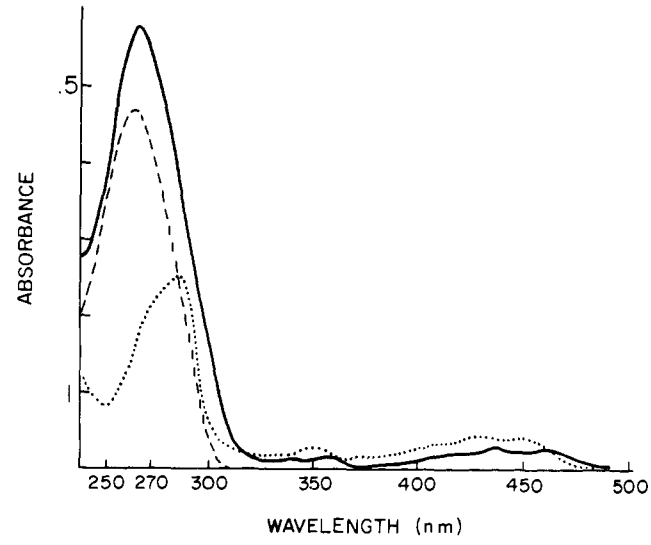

Figure 3. Absorption spectra of: (- - -) DNA (7.2×10^{-5} M); (···) quinacrine (4.5×10^{-6} M); (—) DNA-QAC complex (same concentrations as above) in cacodylate buffer (0.01 M, pH 7).

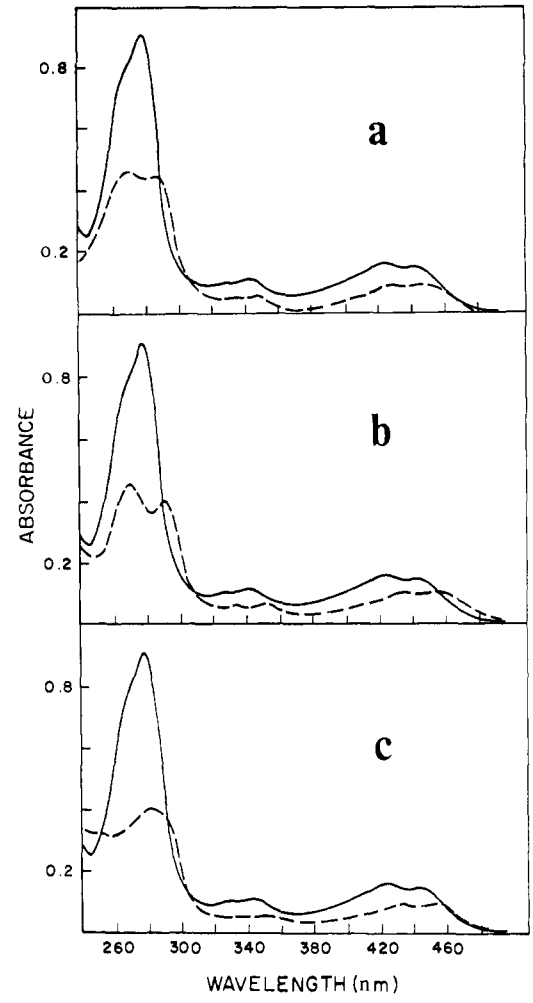

Figure 4. Quinacrine-polynucleotide difference absorption spectra. (—) free quinacrine (1.67×10^{-5} M); (- - -) quinacrine (1.67×10^{-5} M) and polynucleotide (3.1×10^{-4} M) in cacodylate buffer (0.01 M, pH 7). (a) Calf thymus DNA; (b) poly(dA-dT)·poly(dA-dT); (c) poly(dG)·poly(dC). The reference cell contained a concentration of polynucleotide equal to that in the sample cell which contained the quinacrine-polynucleotide solution.

same bands in the spectrum of the free dye by 490 and 500 cm^{-1}, respectively, with negligible change in their Franck-

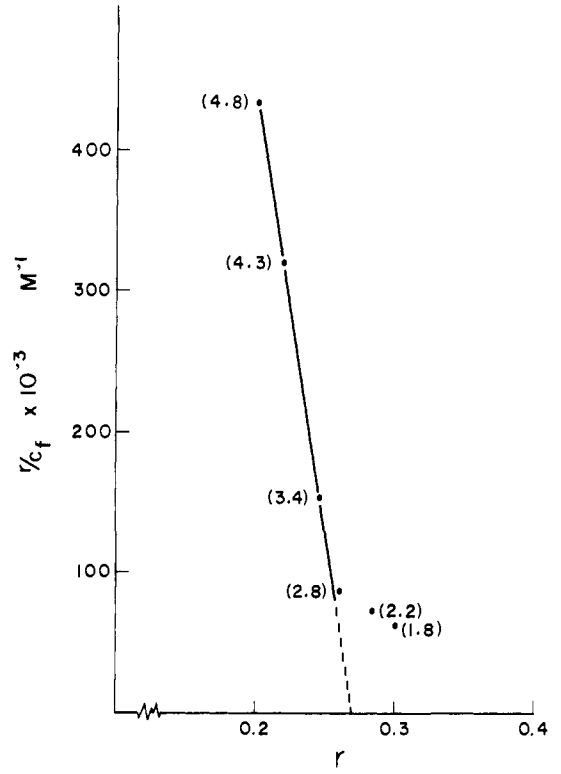

Figure 5. Scatchard plot of quinacrine-DNA complexes. [QAC] = 1.02×10^{-5} M; [DNA] varied from 1.8×10^{-5} to 5.4×10^{-5} M in cacodylate buffer (0.01 M, pH 7). r is the ratio of the concentrations of bound quinacrine to total DNA in the solutions. C_f is the concentration of free quinacrine. Concentrations of bound and free quinacrine were determined from absorbance at 420 nm. Numbers in parentheses are the P/D ratios.

Condon envelopes or vibronic structure. Thus these two lower energy transitions show dispersion shifts only.

However, in the 250–300-nm region two bands are apparent in the difference spectra, with peaks at 290 (34 480 cm^{-1}) and 270 mn (37 040 cm^{-1}). If these two bands are decomposed into two symmetrical Lorentzian components, we obtain two bands with peaks at 34 480 and 37 540 cm^{-1}. The energy difference between these two peaks, Δ = 3060 cm^{-1}, is thus 310 cm^{-1} greater than that between the unperturbed interacting states, $\Delta E_{S_3\text{-}S_1}$ (38 460 − 35 710 cm^{-1}) = 2750 cm^{-1}. Relative to an area of 1.08 for the oscillator strength of the $S_0 \rightarrow S_3$ transition of the free dye, we observed areas of 0.40 and 0.30 for the 290 and 270 nm components, respectively, for the difference spectrum of the complex.

The difference spectrum of QAC bound to polyd(A-T)·polyd(A-T) is shown in Figure 4B. As observed in the QAC-DNA system, the low energy transitions of the free dye are merely shifted to lower energy, whereas in the $S_0 \rightarrow S_3$ region (280 nm) two bands appear at 37 040 (270 nm) and 34 130 cm^{-1} (293 nm). Decomposing this poorly resolved spectral region into two Lorentzian bands as before gives rise to two bands at 37 315 and 34 015 cm^{-1}, and thence Δ = 3300 cm^{-1} again larger than $\Delta E_{S_3\text{-}S_1}$ by 550 cm^{-1}. In direct contrast to these, QAC-DNA and QAC-poly(dA-dT)·poly(dA-dT) systems, the difference spectrum of QAC-poly(dG)·poly(dC) system shows only one broadened band in the QAC 280-nm absorption region, Figure 4c, with a maximum of 35 460 cm^{-1} (282 nm).

Effect of P/D Ratio. A study of the effect of P/D ratio allowed further characterization of the spectral perturbations in the QAC-DNA complexes. The fraction of QAC which remained unbound or free in several complexes of varying P/D ratio was determined by typical Scatchard plot experiments.[5,22] In these experiments the concentration of QAC was kept constant and the DNA concentration was varied. The spectral change at 420 nm was used in the Scatchard representation of the binding isotherm (Figure 5). The limiting visible absorption spectrum obtained at a P/D ratio of ca. 5. The Scatchard plot was clearly biphasic. The apparent number of binding sites per mole of nucleotide (β_{app}) which corresponded to the steep (strong "primary" binding) portion of the curve was 0.27. The association constant K_a obtained from the slope of this part of the isotherm was 6×10^6 M^{-1}. Our values for these binding parameters of QAC and DNA are compared with those previously published by other workers in Table I. The value of 0.27 for β_{app} is consistent with the model of exclusion of adjacent binding sites[23] which predicts a value of 0.25 for β_{app}. The large discrepancy between these results and those of Modest and Sengupta[24] may be because the later workers worked at lower P/D ratios than we did.

Table I. Summary of Binding Parameters of Quinacrine and DNA

	β_{app}	K_a, M^{-1}
Rat liver DNA,[4] 0.01 M Tris-Cl, pH 8	0.051	4×10^5
Calf thymus DNA,[5] 0.1 M NaCl, pH 6.5	0.25	2.55×10^5
Calf thymus DNA,[24] 0.01 M phosphate, pH 7	0.72	1.6×10^7
Calf thymus DNA,[40] 0.01 M cacodylate, pH 7	0.27	5.9×10^6
Poly(dG)·poly(dC),[35] 0.01 M cacodylate, pH 7	0.25	2.6×10^6
Poly(dA-dT)·poly(dA-dT)[40] 0.01 M cacodylate, pH 7	0.29	7.9×10^6

Immediately these experimental results eliminate the possibility that the two bands observed in the ultraviolet region of the difference spectra of QAC-DNA complexes could be due to bound and free QAC since there is negligible free dye at a P/D ratio of 5 and the difference spectra described herein were obtained at a P/D ratio of 20.

The ultraviolet difference spectra at several P/D ratios from 1:1 to 56:1 were measured and it was found that the previously described splitting in the ultraviolet spectral region did not vary appreciably between P/D ratios of 56:1 to 6:1 (Figure 6). As the P/D ratio was further reduced from 5:1 to 1:1, the doublet became a broadened maximum at 280 nm owing to the increasing amount of free dye (vide supra). In fact a clear isosbestic point at 288 nm in this latter P/D range was observed when the experiment was conducted with the QAC concentration kept constant and the DNA concentration varying (Figure 7).

Several other important conclusions which characterize the nature of the complex which results in the observed spectral splitting can be determined from the following treatment of the above data.

If A_T and A_Δ are the total absorbancy, and the difference absorbancy, respectively, at a particular wavelength, λ, then

$$A_T = \epsilon_b C_b + \epsilon_f C_f + \epsilon_p (C_p - C_p') + \epsilon_{p'} C_{p'} \qquad (2)$$

and

$$A_\Delta = A_T - \epsilon_p C_p \qquad (3)$$

where ϵ_b, ϵ_f, $\epsilon_{p'}$, ϵ_p are the molar decadic extinction coefficients of bound dye, free dye, perturbed nucleotide bases, and nucleotide bases in the absence of any dye, respectively,

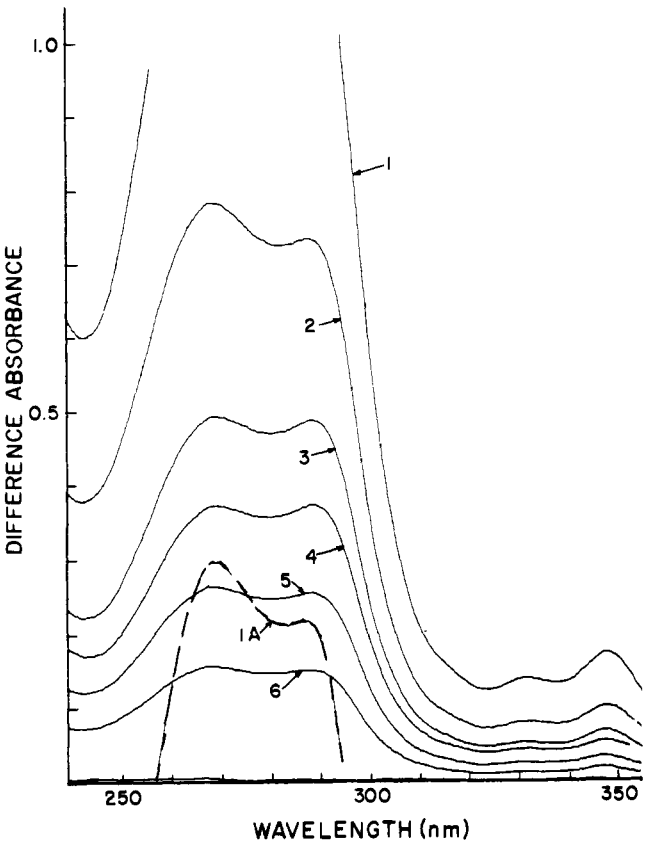

Figure 6. Difference absorption spectra of quinacrine-DNA complexes [DNA] = 3×10^{-4} M; [quinacrine] curves 1 and 1A, 5×10^{-5} M (P/D = 6); curve 2, 3×10^{-5} M (P/D = 10); curve 3, 2×10^{-5} M (P/D = 15); curve 4, 1.5×10^{-5} M (P/D = 20); curve 5, 1×10^{-5} M (P/D = 30); curve 6, 6×10^{-6} M (P/D = 50). Absorbance scale for curve 1A is 1-2.

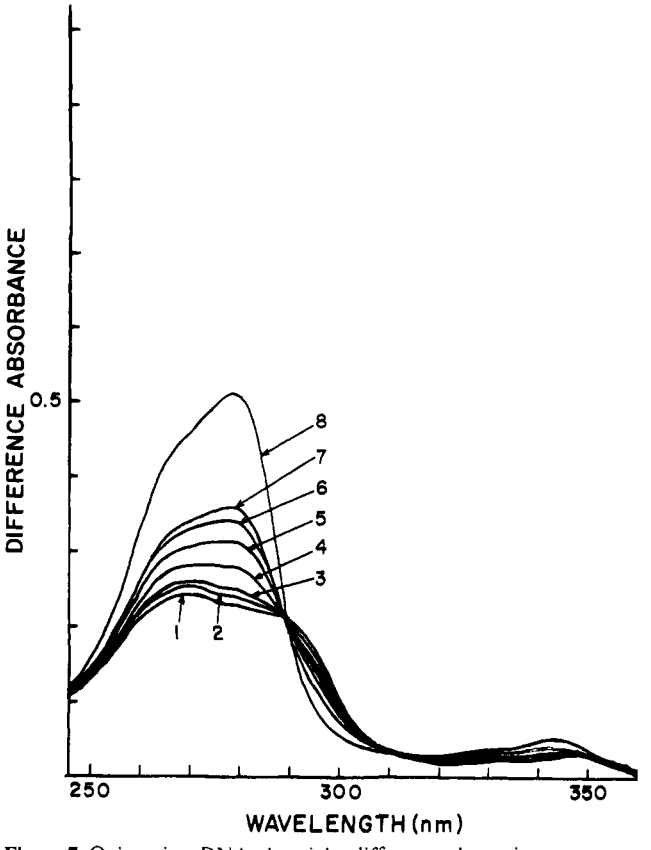

Figure 7. Quinacrine-DNA ultraviolet difference absorption spectra at several P/D ratios. [QAC] = 1.02×10^{-5} M. [DNA]: curve 1, 5.4×10^{-5} M (P/D = 5.3); curve 2, 4.9×10^{-5} M (P/D = 4.8); curve 3, 4.4×10^{-5} M (P/D = 4.3); curve **4**, 3.5×10^{-5} M (P/D = 3.4); curve 5, 2.8×10^{-5} M (P/D = 2.75); curve 6, 2.2×10^{-5} M (P/D = 2.2); curve 7, 1.8×10^{-5} M (P/D = 1.8); curve 8, 0. All solutions in cacodylate buffer (0.01 M, pH 7).

and C_b, C_f, $C_{p'}$, C_p their respective concentrations. In the P/D range of 6:1 to 56:1, according to the binding isotherm experiment, we can make the reasonable assumption that there is a direct relationship between $C_{p'}$ and C_b:

$$C_{p'} = \gamma C_b \tag{4}$$

Hence at any wavelength

$$A_\Delta = C_b[\epsilon_b + \gamma(\epsilon_{p'} - \epsilon_p)] + \epsilon_f C_f \tag{5}$$

Additionally at P/D ratios greater than 6, C_f is negligible and

$$C_b = C_T \tag{6}$$

where C_T is the total dye concentration. Plots of A_Δ vs. C_T at several wavelengths in the ultraviolet region were linear. The plots (Figure 8) at two wavelengths, 260 and 280 nm, should be the most sensitive to the assumptions used in the above treatment and hence their linearity or lack thereof would affect the validity of any conclusions which might be drawn from them. As Figure 8 shows, these plots were linear over the whole P/D range of 6:1 to 56:1. This suggests first, that γ is constant, indicating that there is only one spectroscopically distinct species, which corresponds to the strong primary binding process in this P/D range. The isobestic point referred to previously in Figure 5 confirms this conclusion.

Secondly, ϵ_b must be constant indicating that dye-dye interactions are negligible. Further, the constant shape of the Franck-Condon envelope of the difference spectra over this P/D range shows that the observed splitting is not the result of dye-dye interactions. This conclusion is reinforced by the

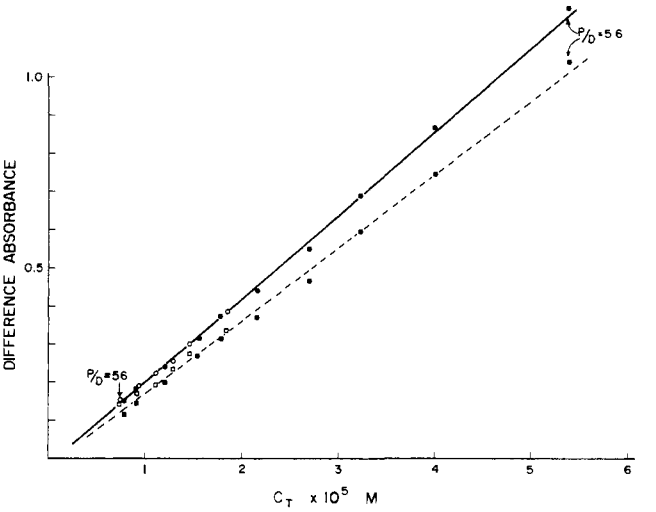

Figure 8. Plot of difference absorbance (A_Δ) of quinacrine-DNA complexes against total quinacrine concentration (C_T). (Circles) 280 nm; (squares) 260 nm. (Open circles and squares) [DNA] = 4.1×10^{-4} M; (closed circles and squares) [DNA] = 3.0×10^{-4} M.

previous conclusion of there being only one type of binding. The weak secondary binding of a second QAC molecule to one which is already bound must be insignificant at P/D ratios greater than 6.

As well $\epsilon_{p'}$ must be constant and hence the observed splitting results from highly localized interactions between the dye molecule and their nearest neighbor. If the interaction were otherwise, a variation in $\epsilon_{p'}$ would be expected.

One additional explanation may be that the splitting results because of the existence of two electronic transitions in this spectral region of QAC which become more distinct on binding. This has already been eliminated since the polarized fluorescence excitation spectrum of QAC (Figure 2) clearly indicated there was only one electronic transition between 260 and 300 nm.

Finally, the observed splitting cannot be due to a change on binding in the extent of protonation of QAC. The spectrum of the conjugate base of QAC at pH 9 includes only a broad structureless absorption maximum at 418 nm in the visible spectral region, whereas the visible spectrum of the bound form includes only a dispersion shifted band at 455 nm.

In summary, the splitting in the ultraviolet difference spectra of QAC–DNA and QAC–poly(dA-dT)·poly(dA-dT) complexes (a) results from nearest neighbor interactions of the nucleic acid bases and QAC, (b) is characteristic of only one mode of binding, the strong "primary" binding process, (c) is not the result of dye–dye interactions and (d) is not observed in QAC–poly(dG)·poly(dC) complexes, although the visible absorption spectra of all these QAC–polynucleotide complexes are superimposable.

Spectroscopic Assignment. These conclusions together with the dramatic difference in the 280-nm region between the spectra of free QAC and QAC bound to DNA or poly(dA-dT)·poly(dA-dT) (i.e., the large intensity redistribution and pronounced band shifts and splitting) is good evidence that the interaction between the S_3 state of QAC and its environment is not simply due to a dispersion shift.

At present the splitting in the ultraviolet difference spectrum of the complex can reasonably be attributed to dipole–dipole interactions between neutral exciton states of the complex (states corresponding to the S_3 state of the dye, QAC, and the S_1 state of the nucleic acid bases). The result of preliminary theoretical calculations[25] while based on a number of simplifying assumptions is not inconsistent with this assignment. These calculations suggested that additional interactions between the exciton states and charge resonance states of the complex should also be considered to fully account for the observed splitting.

Regardless of the precise theoretical model for the electronic exchange interactions which result in the observed splitting in the difference spectra of QAC–DNA and QAC–poly(dA-dT)·poly(dA-dT) complexes, the experimental results indicate that there is an important difference in the binding of QAC to poly(dG)·poly(dC) compared with these other polynucleotides. Neither the visible absorption spectra (350–500 nm) nor the fluorescence spectra allow this distinction.

One can visualize, even by the simple physical consideration of the r^{-3} dependence of the interaction energy, that maximum dipole–dipole interaction would be observed when the dye and nucleic acid nearest neighbors are arranged to allow maximum overlap of the interacting molecules. Therefore these results indicate that the distance of approach between QAC and the G-C base pairs is much larger than that between QAC and the A-T base pairs in the polynucleotides studied. This interpretation is reasonable since the more tightly wound poly(dG)·poly(dC) helix is less likely to unwind than the other two polynucleotide helices in order to accommodate the intercalating dye molecule. The work of Ramstein and Leng[30] on the contour lengthening of DNAs of different base composition when complexed with proflavin also indicated that this dye was bound more externally to G-C rich regions of DNA. The fluorescence quenching experiments (vide infra) substantiate this concept. The position of the dye for the closest approach to a base pair will be that when the dye molecule is intercalated between the planes of the base pairs of the double helix. This interpretation is also consistent with the result that the spectral splitting would be observed to a limiting ratio of 2.5 base pairs per dye (P/D = 5). The different difference spectra observed for poly(dA-dT)·poly(dA-dT) and DNA–dye complexes and on the one hand, the poly(dG)·poly(dC)–dye complex on the other supports the intercalation model in the former two polynucleotides.

Polarization Spectra. Evidence for the exciton-like origin of the splitting in QAC–DNA and QAC–poly(dA-dT)·poly(dA-dT) complexes might be expected from their polarized fluorescence excitation spectra. In the absence of higher order configuration interaction, it would be expected that the 445- and 344-nm bands of the excitation spectrum would be polarized in the same direction in the complexed dye as in the free dye. Whereas, if exciton interaction were occurring, the two bands at 270 and 290 nm observed in the difference spectrum of the complex could be polarized at an angle as large as 90° to each other depending on the geometry of the complex and at some angle to the polarization of the 280-nm band in the spectrum of the free QAC. However, this wavelength region is experimentally difficult and is further complicated by the fact that singlet–singlet energy transfer[31] from the nucleotide to the bound dye occurs. At the P/D ratios at which we worked (P/D = 20), the nucleotide bases absorb the greater portion of the exciting light. The polarized fluorescence excitation spectrum of a QAC–poly(dA-dT)·poly(dA-dT) complex (cacodylate buffer) is shown in Figure 2. The observed polarization of the 445-nm band, while positive, was lower than that of the free dye. However, the polarization spectrum for the complexed dye was measured in aqueous solution while the polarization of the free dye was measured in a viscous glycerol solution and this could readily account for this apparent depolarization. The emission resulting from excitation in the 250–300-nm region was essentially depolarized, a result which was not unexpected in view of the above comments.

Fluorescence Studies. Other experimental techniques are available to probe the nature of this dye–polynucleotide binding. For example, the intercalation model further suggests that in these QAC–DNA, quinacrine–poly(dA-dT)·poly(dA-dT) systems the dye molecules are sterically protected from collisions with other molecules in solution. This steric protection should be mirrored in the magnitude of quenching of dye fluorescence by an additive in the solution. Halide ions are known to quench the fluorescence of a large number of aromatic compounds; the large polarizable iodide ion is known to be particularly effective in this regard as was demonstrated by Oster[32] in his pioneering work on the quenching of dye fluorescence by nucleic acids. The quenching is presumably caused by the formation of an excited-state, charge-transfer complex between the halide ion and the excited aromatic molecule. For this reason we have studied the fluorescence quenching of these same QAC–polynucleotide systems to study the accessibility of the bound dye to the iodide ion in solution.

A number of workers have reported that iodide ion does not quench the fluorescence of dyes complexed to nucleic acids, and they attributed this lack of quenching to the repulsion of the negatively charged iodide ion by the polyanion DNA.[33,34] This property has even been used to calculate the binding characteristics of proflavin, assuming that only the free dye fluorescence is quenched by the iodide ion.[34]

In the case of QAC we have shown that the complexed dye fluorescence can be quenched by iodide, and that, as expected, the extent of quenching depends on the nature of the nucleic acid used (Table II).

To obtain the fluorescence quenching rate constants (k_q),

Table II. Quenching Rate Constant of Free Dye and Dye-Nucleic Acids Complexes Fluorescence by Iodide Ion[a]

	$(I^0/I)_f$	$A = I_f^0/I_{f+b}^0$	$(I^0/I)_{f+b}$	$(I^0/I)_b$	τ, ns	$k_q \times 10^{-9}$ M^{-1} s^{-1} uncorr	$k_q \times 10^{-9}$ M^{-1} s^{-1} corr
QAC	4.1	1			4		7.8
QAC + poly(dA-dT)·poly(dA-dT)		0.4	1.4	1.38	20	0.20	0.19
QAC + poly(dG)·poly(dC)		7.0	2.8	2.38	6	3.0	2.3
QAC + native DNA		2.1	2.2	2.09	10	1.2	1.1

[a] Maximum iodide concentration: 0.1 M, P/D = 20, [QAC] = 2×10^{-5} M.

we have measured fluorescence intensities as a function of iodide concentration with $[I^-] + [Cl^-] = 0.10$ M (to maintain constant ionic strength), P/D ratio of 20, and [QAC] = 2×10^{-5} M. We have computed the quenching rate constants from the slope of the well-known Stern-Volmer plot[35] using singlet lifetimes measured on a TRW fluorescence lifetime apparatus. In all cases studied, linear Stern-Volmer plots were observed. A correction was applied to the observed k_q values in order to ensure that the differences observed were not merely due to a small amount of free dye which may still be present even at high P/D ratios.[33] Any free dye would have a larger contribution to the emission of the poly(dG)·poly(dC)-QAC complex since the dye fluorescence is quenched on binding in this case while it is enhanced in the case of the poly(dA-dT)·poly(dA-dT)-QAC complex.

Let I_f and I_b be the relative fluorescence efficiencies of the free and bound form, respectively; then the fluorescence efficiency of the mixture of free and bound dye is given by:

$$I_{f+b}^0 = \alpha I_b^0 + (1 - \alpha) I_f^0 \quad (7)$$

at zero iodide concentration, and

$$I_{f+b} = \alpha I_b + (1 - \alpha) I_f \quad (8)$$

in the presence of iodide where α is the fraction of bound dye and the superscript zero designates the fluorescence at zero iodide concentration. Hence:

$$(I^0/I)_b = [1 - (1 - \alpha)A]/[(I^0/I)_{f+b}^{-1} - (1 - \alpha)A(I^0/I)_f^{-1}] \quad (9)$$

where $A = I_f^0/I_{f+b}^0$ is the observed ratio of the fluorescence efficiencies of free dye to free plus bound dye, and $(I^0/I)_{f+b}$ is the observed quenching efficiency. $(I^0/I)_b$ is the corrected quenching efficiency. We allowed a correction in which we estimated the concentration of the free dye in these complexes as being 5% of the total dye concentration. Scatchard plot results indicate that this was a reasonable estimate of free dye in the DNA-QAC complexes at P/D ratios greater than 20 ($\mu = 0.1$). In addition the visible absorption spectra of the poly(dA-dT)·poly(dA-dT)-QAC and poly(dG)·poly(dC)-QAC complexes were superimposable on that of the DNA-QAC complex at P/D = 20.

We found that there was no significant difference between the corrected and uncorrected values of k_q. Only a gross underestimation of the amount of free dye present would have invalidated our conclusions.

The results reported in Table II show that the fluorescence of QAC bound to various nucleic acids can be quenched by iodide, in contrast to the results obtained with other dyes.[33,34] It has been shown that fluorescence quenching by iodide is diffusion controlled so that binding of the dye to the polynucleotide, by decreasing the diffusion rate of the excited species, would be expected to limit the quenching efficiency.

The value of k_q (7.8×10^9 M^{-1} s^{-1}) determined for the free dye is in agreement with the calculated diffusion-controlled value in water.[36] The lowest k_q value was observed for the QAC-poly(dA-dT)·poly(dA-dT) complex, and the highest for the QAC-poly(dG)·poly(dC) complex. Since A-T rich polynucleotides have a more open structure than those rich in G-C, one would have expected a higher value for k_q in the former complex compared with the latter complex. The fact that the opposite was observed reinforces our conclusion that the bound dye was less accessible in the QAC-poly(dA-dT)·poly(dA-dT) complex. These observations confirm our interpretation of the spectroscopic experiments described above; that is, the difference in splitting observed for the two complexes originates from closer binding of QAC to A-T than to G-C base pairs in the polynucleotides studied.

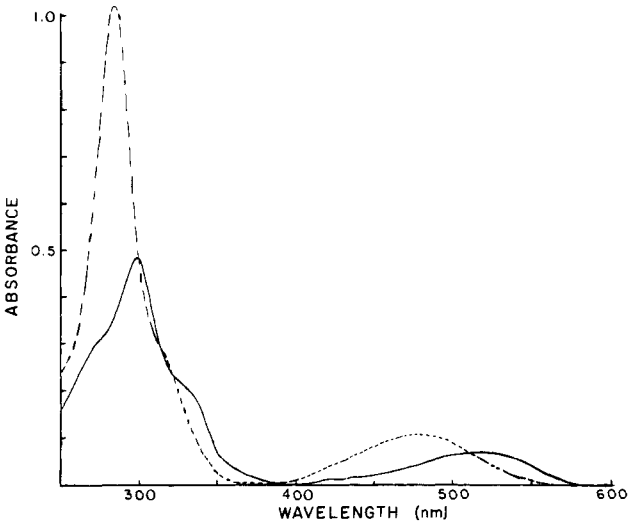

Figure 9. Absorption spectrum of ethidium bromide (2.0×10^{-5} M). (- - -) difference absorption spectrum of ethidium bromide (2.0×10^{-5} M)-DNA (2.85×10^{-4} M) complex; (—) in cacodylate buffer (0.01 M, pH 7).

Ethidium Bromide-DNA Complex. We have also measured the ultraviolet difference spectrum on complexes of ethidium bromide (EB) and DNA. This spectrum (P/D = 14, 0.01 M cacodylate buffer, pH 7), together with that of free EB, is shown in Figure 9. The ultraviolet difference spectrum of EB-DNA is characterized by two bands at 272 and 298 nm with a broad shoulder at 300 nm and marked intensity reduction compared with the absorption of free EB which has an intense maximum at 285 nm with a weak shoulder at 315 nm. The difference spectrum of EB-DNA complex which we observed is similar to that recently published by Houssier and co-workers[37] and earlier by Sutherland.[38] The former workers, in their detailed study, on the optical and electro-optical spectra of EB-DNA complexes, and Williams and Seligy[39] in their study on the circular dichroism spectra of EB-polynucleotide complexes offered

several alternative interpretations of the origin of their circular dichroism spectra. Our interpretation that the bands in the ultraviolet difference absorption spectra may arise from an exciton-like interaction has been suggested as a possible explanation of the circular dichroism spectra in the same spectral range.

Summary

We have observed two bands and marked intensity redistribution in the ultraviolet difference absorption spectra (260–300 nm) of QAC-poly(dA-dT)·poly(dA-dT) and QAC-DNA complexes but not in QAC-poly(dG)·poly(dC) complexes. In contrast the visible absorption spectra of these complexes were all superimposable.

We have shown that these ultraviolet spectral perturbations are due to nearest neighbor interactions between QAC and the nucleic acid bases and are not due to dye-dye interactions. The splitting is also characteristic of only one type of binding process, the strong, intercalative binding. We have also shown that there is only one electronic transition in the 260–300-nm region of the absorption spectrum of QAC. We therefore conclude that a reasonable interpretation of the observed splitting in the QAC-polynucleotide complexes is that it is due to an interaction between neutral exciton states and, quite possibly, charge resonance states of the QAC-polynucleotide complex. This interaction occurs when the dye molecule is intercalated into the polynucleotide double helix.

Therefore, the observation of splitting in the ultraviolet spectral region is dependent on the distance of approach between the dye and the nucleic acid bases. QAC is bound considerably closer to the nucleic acid base pairs in DNA and poly(dA-dT)·poly(dA-dT) than to G-C base pairs in poly(dG)·poly(dC). Theoretical calculations are consistent with this interpretation and quenching experiments of QAC fluorescence by iodide ions confirm the structural dependence of the spectral effect. We believe that splitting in the ultraviolet difference absorption spectra of dye-nucleic acid complexes can provide important additional information on the structure on these complexes and, since it is characteristic of the intercalative binding process, it can be used as a diagnostic for helix-coil transitions.

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