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Binding of *N*-Substituted Anthracenecarboxamides to Double-Stranded DNA: An Electronic Spectral Study

STEPHEN G. SCHULMAN [x], W. LARRY PAUL [*], and ROY J. STURGEON [‡]

Abstract □ The electronic absorption and fluorescence spectra of two isomeric anthracenecarboxamides substituted with quaternary alkylammonium groups were studied as a function of solvent polarity, solvent rigidity, and state of protonation. These relatively simple environmental perturbations of the electronic spectra were employed to interpret spectral shifts caused by complexation with the bases or base pairs of DNA. The exocyclic side chains of the compounds studied have some freedom of movement when the spectroscopic probes are bound to double-stranded DNA.

Keyphrases □ Anthracenecarboxamides, *N*-substituted—binding to DNA, electronic absorption and fluorescence spectral study □ Binding—*N*-substituted anthracenecarboxamides to DNA, electronic absorption and fluorescence spectral study □ DNA—binding to *N*-substituted anthracenecarboxamides, electronic absorption and fluorescence spectral study □ Electronic absorption spectrometry—binding of *N*-substituted anthracenecarboxamides to DNA □ Fluorescence spectrometry—binding of *N*-substituted anthracenecarboxamides to DNA

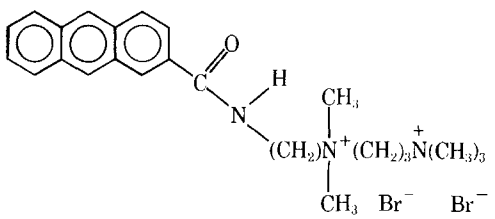
Many drugs active against microorganisms and neoplasms are polycyclic aromatic or heteroaromatic cations, which bind reversibly to the polyanionic nucleic acids *in vitro* and *in vivo* (1). Their modes of action are generally believed to be related, directly or indirectly, to their ability to be bound to DNA, resulting in errors of transcription or replication in the affected organism (1). However, the process of binding to DNA often entails interactions different from, and in addition to, the electrostatic interaction between ions of opposite charge.

Various techniques such as low-shear viscometry, ultracentrifugation, and NMR spectroscopy have yielded information indicating that the aromatic portions of the drugs can become inserted (intercalated) between base pairs of the double helix of DNA. This insertion results in a partial unwinding and linear extension of the double helix to accommodate the intruding drug molecule (2–5).

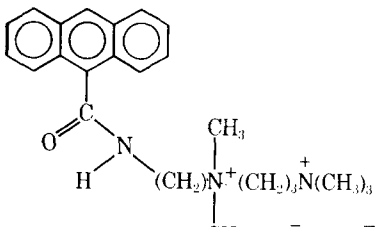
Several studies of binding believed to result in intercalation of drug or dye molecules with DNA have been carried out, with the binding parameters often estimated absorptiometrically or fluorometrically. However, little has been established concerning the nature of the changes in the electronic absorption or fluorescence spectra of the drug or dye molecules upon such binding. The failure of electronic spectroscopy to deliver useful information about nucleic acid binding has been due in part to the complex structures of the drug and dye molecules involved; the environmental perturbations of these spectra are difficult to interpret.

In the present study, the changes in the electronic absorption and fluorescence spectra of two cationic, *N*-substituted anthracenecarboxamides occurring upon binding to calf thymus DNA were examined. The observed changes were compared to the changes occurring in the spectra of these compounds upon protonation and as a function of solvent rigidity and polarity.

The compounds studied, *N*-(2-anthroyl)-*N'*,*N'*-dimethyl-*N'*-[(3-trimethylammonium)propyl]ethylenediamine dibromide (I) and *N*-(9-anthroyl)-*N'*,*N'*-di-



I



II

methyl-*N'*-[(3 - trimethylammonium)propyl]ethylenediamine dibromide (II), are representatives of a class of doubly charged cations that increase the intrinsic viscosity of solutions of double-stranded DNA upon binding and, therefore, are presumed to intercalate between DNA base pairs (6). These molecules were chosen as probes because they absorb and fluoresce at long wavelengths where the electronic spectra of the purine and pyrimidine bases of DNA do not interfere and because their aromatic systems are functionally simple, each containing a single electron acceptor group (the carboxamido group) that can affect the electronic spectra.

EXPERIMENTAL

Calf thymus DNA[1], supplied as the A grade sodium salt (phosphorus content 8.04% by weight) was dissolved in water containing monobasic and dibasic potassium phosphate as a buffer system (pH 6.9, total potassium-ion concentration of 0.01 *M*) to make a stock solution 1.10×10^{-2} *M* in DNA phosphate. Spectroquality *n*-heptane[2] and analytical reagent grade sulfuric acid[3] also were employed as solvents. Sulfuric acid solutions were prepared by dilution with distilled deionized water. The Hammett acidity scale of Jorgensen and Hartter (7) was employed to calibrate the acid solutions.

Absorption spectra of aqueous buffer and sulfuric acid solutions, $\sim 1 \times 10^{-4}$ *M* in I and II, were taken on a grating spectrophotometer[4]. When the probes were to be complexed by DNA, 200 μl of the DNA stock solution was added to 2 ml of a buffered, aqueous 1×10^{-4} *M* solution of I or II in 1-cm square silica cells. This mixture produced a large ratio (~10) of DNA phosphate to probe concentration, which was necessary to maximize the binding of the probe in the region where intercalation may be expected to predominate ([DNA phosphate] > 4 × [probe]) and to minimize the binding of the probe to the external parts of the DNA double helix. Because of the very low solubility of I and II in *n*-heptane, all spectra in that solvent were taken on solutions saturated with I and II.

Fluorescence spectra were taken on a fluorescence spectrophotometer[5] with a rhodamine-B quantum counter[5]; the monochromators were calibrated against the xenon line emission spectrum, and the spectral output was corrected for a wavelength variable response. Because of the nonlinearity of the fluorescence intensity with varying concentration at high absorbances, the aqueous and acid solutions upon which fluorescence spectra were taken were kept to $\sim 5 \times 10^{-6}$ *M* in I or II. However, the DNA phosphate concentration in the aqueous solutions in which the DNA complexes of I and II were prepared for fluorometric study was identical to that employed in the absorptiometric experiment.

[1] Calbiochem, La Jolla, Calif.
[2] Matheson, Coleman and Bell, East Rutherford, N.J.
[3] Mallinckrodt Chemical Works, St. Louis, Mo.
[4] Model DB-GT, Beckman Instruments, Fullerton, Calif.
[5] Model MPF-2A, Perkin-Elmer, Norwalk, Conn.

Table I—Electronic Absorption (λ_a) Maxima of I and II in Water with and without DNA, in 90% H_2SO_4, and in *n*-Heptane, All at 298 °K

In Water, pH 6.9						
Without DNA		With 1×10^{-3} *M* DNA		In 90% H_2SO_4 without DNA		In *n*-Heptane[a] without DNA,
λ_a, nm	log ε	λ_a, nm	log ε	λ_a, nm	log ε	λ_a, nm
			I			
386	3.36	398	3.29	422	3.43	383
371	3.39	382	3.32	—	—	364
358	3.43	365	3.30	363	3.42	348
340	3.40	346	3.31	347	3.65	338
328	3.25	~330	3.24	330	3.52	~327
			II			
383	3.75	390	3.49	384	3.63	381
364	3.81	371	3.55	363	3.64	362
346	3.64	353	3.43	348	3.47	344
331	3.38	337	3.23	333	3.26	328
318	3.08	~322	3.23	~319	3.15	~313

[a] Absorption spectra in *n*-heptane are only qualitative because of the poor solubilities of I and II in that solvent.

The quantum yields of fluorescence of the free and complexed probes were determined by the comparative method of Parker and Rees (8), using quinine bisulfate in 0.1 *N* H_2SO_4 as a standard. The absorbances of the solutions of I and II and those of the quinine standard solution were less than 0.02 at the wavelength of excitation. This finding circumvented the necessity for corrections to the integrated fluorescence intensity measurements for the nonlinearity at high absorbances (8).

Fluorescence decay times (lifetimes of the lowest excited singlet states) of the free and DNA-bound probes were measured on a pulsed-source fluorometer[6] with an 18-w deuterium lamp[6]. A pulse time of 1.6 nsec and an internal computer[7] were used to resolve the lamp pulse characteristics from the probe decay characteristics. The unit was interfaced with a dual-beam oscilloscope[7] with two 1A2 plug-in amplifiers[7] for visual display of the fluorescence decay characteristics.

RESULTS AND DISCUSSION

The principal absorption and fluorescence features of I and II and their variations with the state of protonation, solvent polarity, and solvent rigidity are listed in Tables I and II. The absorption spectra of I and II in water, concentrated sulfuric acid, and aqueous DNA solutions are shown in Figs. 1 and 2, and their fluorescence spectra are shown in Figs. 3 and 4.

The absorption spectra of substituted anthracenes in the 300–450-nm region are derived from the absorptions from the ground state (^1A) to the two lowest lying excited states (^1L_a and ^1L_b) (9). In anthracene, the $^1L_a \leftarrow {}^1A$ and $^1L_b \leftarrow {}^1A$ transitions, which are short axis and long axis polarized, respectively (9), overlap considerably and give rise to the structured absorption band complex at 300–400 nm. Although the two absorption bands are indistinguishable in anthracene, it is generally believed that the long wavelength edge of the absorption complex belongs to the ^1L_a band (10) and that the ^1L_b band is slightly higher in energy, much less intense than the ^1L_a band, and, therefore, buried under the broad absorption envelope of the latter (11).

Introduction of an electron donor or acceptor substituent to the β-position of anthracene is generally presumed to affect the longitudinally polarized $^1L_b \leftarrow {}^1A$ transition of the anthracene ring to a greater degree than the transversely polarized $^1L_a \leftarrow {}^1A$ transition (9). Therefore, the ^1L_b band of the 2-substituted anthracenes is red shifted, intensified, and blurred to a greater extent than the ^1L_a band (11–14). A strongly interacting substituent in the 2-position may, in theory, cause the ^1L_b band to move to a lower energy than the ^1L_a band of the same compound and, hence, become the longest wavelength absorption band.

The absorption spectra of I demonstrate considerable blurring, especially on the long wavelength side, relative to the absorption spectrum of anthracene. No doubt this blurring results from the intensification and blurring of the $^1L_b \leftarrow {}^1A$ transition. However, whether or not the ^1L_b band lies at a lower energy than the ^1L_a band cannot be established by simple inspection because of substantial overlap. However, when the amide group is protonated in concentrated sulfuric acid, the unstructured band that moves to much longer wavelengths (427 nm) can be assigned

[6] Model 31B, TRW, Inc., El Segundo, Calif.
[7] Model 556, Tektronix, Inc., Beaverton, Ore.

Table II—Fluorescence (λ_f) Maxima of I and II in Water with and without DNA at 298 °K (Fluid) and at 77 °K (Rigid) in 90% H_2SO_4 and in *n*-Heptane at 298 °K along with the Fluorescence Quantum Yields (ϕ_f) and Lifetimes (τ_f) of the Excited Free and DNA-Bound Probes

In Water, pH 6.9, T = 298 °K		In Water, pH 6.9, T = 77 °K		In 90% H_2SO_4, T = 298 °K,	In *n*-heptane, T = 298 °K,
Without DNA	With 1×10^{-3} *M* DNA	Without DNA	With 1×10^{-3} *M* DNA	without DNA	without DNA
			I		
λ_f (nm) = 452 (max) ϕ_f = 0.22 τ_f = 23.1 nsec	λ_f (nm) = 454 (max) ϕ_f = 0.18 τ_f = 16.6 nsec	λ_f (nm) = 415, 437 (max), and 456	λ_f (nm) = 422, 444 (max), and 465	λ_f (nm) = 524 (max)	λ_f (nm) = 390 (max), 414, and 439
			II		
λ_f (nm) = 400 and 417 (max) ϕ_f = 0.03 τ_f = 3.0 nsec	λ_f (nm) = 437 (max) ϕ_f = 0.14 τ_f = 21.5 nsec	λ_f (nm) = 392 (max) and 414	λ_f (nm) = 398 and 419 (max)	λ_f (nm) = 480 (max)	λ_f (nm) = 388 and 424

to the $^1L_b \leftarrow {}^1A$ transition. The highly structured absorption band of the protonated probe is only slightly red shifted relative to the corresponding vibrational features in the unprotonated probe and corresponds to the $^1L_a \leftarrow {}^1A$ transition. A small blue shift of the absorption spectrum of I is observed going from water to *n*-heptane, reflecting the lower polarity of the hydrocarbon solvent, which destabilizes the highly polar excited states involved in absorption relative to the aqueous solvated excited states (15).

The fluorescences of I and its conjugate acid in fluid aqueous solutions are unstructured and occur far downfield from the corresponding absorption spectra (*i.e.*, they are highly Stokes shifted). As in the absorption spectrum, the fluorescence of the protonated form lies at much longer wavelengths than that of I itself. In frozen aqueous media and low dielectric media, the fluorescence spectrum of I blue shifts dramatically and becomes highly structured relative to the fluorescence spectrum in fluid water. By freezing the aqueous solution of I to rigidity, the molecule is "locked" into the equilibrium ground-state geometrical and solvent configurations. As a result of locking the fluorescing molecule into the ground-state geometry and solvent cage, fluorescence originates at a higher potential energy than it would under fluid conditions, causing the blue shift upon freezing. The elimination of thermal motion of the functional group and the solvent cage by freezing results in quantization of the vibrational structure of the ground state and, hence, the highly structured spectrum.

In heptane, the nonpolar solvent interacts very weakly with the ground-state or excited molecule (16), so that the effect of solvent relaxation in the excited state and of thermal motion of the ground-state solvent cage, even in fluid heptane, is small. Therefore, there is a higher energy and a higher degree of vibrational structure in the fluorescence of I in heptane relative to that in water.

In anthracenes substituted in α-positions (1- and 9-positions), the transversely polarized $^1L_a \leftarrow {}^1A$ transition is more affected by substituents and environmental perturbations than is the longitudinally polarized $^1L_b \leftarrow {}^1A$ transition. As a result, the ^1L_a band loses vibrational structure and moves to longer wavelengths relative to its position in anthracene while the ^1L_b band retains structure and is only slightly affected (11–13). However, a qualification must be placed on this general statement.

In 9-substituted anthracenes where the substituent groups are relatively bulky (*e.g.*, COO^-, COOH, COOR, and $CONH_2$), steric hindrance arising from interaction between the bulky substituent in the 9-position and the peri hydrogen atoms in the 1- and 8-positions of the anthracene ring prevents the conjugation (coplanarity) of the exocyclic group in the 9-position with the anthracene ring (17, 18). In this case, the 9-substituent produces a weak perturbation and results in absorption spectra almost identical with those of anthracene: highly structured, shifted slightly to longer wavelengths, and insensitive to changes in the electric field produced by varying the dielectric strength of the solvent or the state of protonation of the exocyclic group (16–19). However, in the ^1L_a state, the charge distribution is more stabilized by conjugation of the exocyclic group with the anthracene ring than in the ground state and the gain of

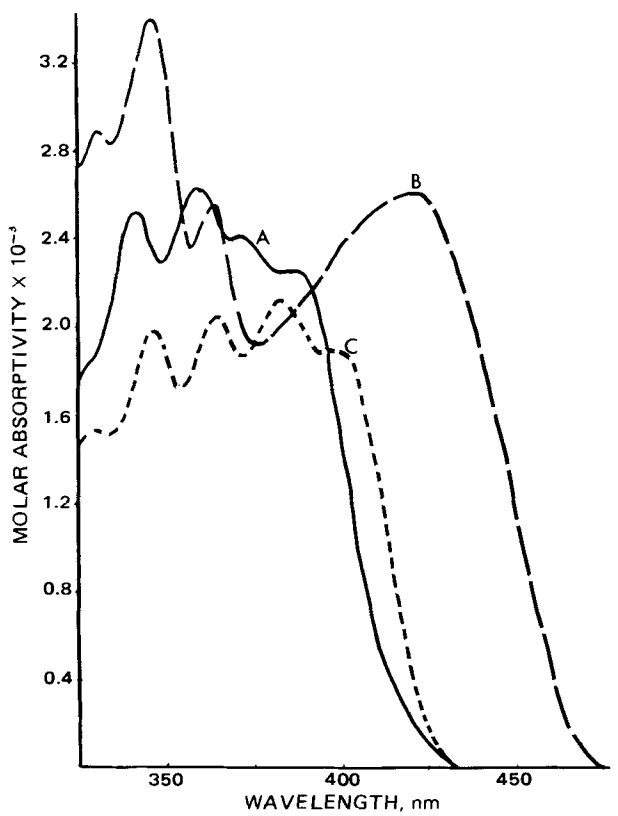


Figure 1—*Electronic absorption spectra of the spectroscopic probe I in aqueous solution (A), in concentrated sulfuric acid (B), and bound to DNA (C). All spectra were taken at 298 °K.*

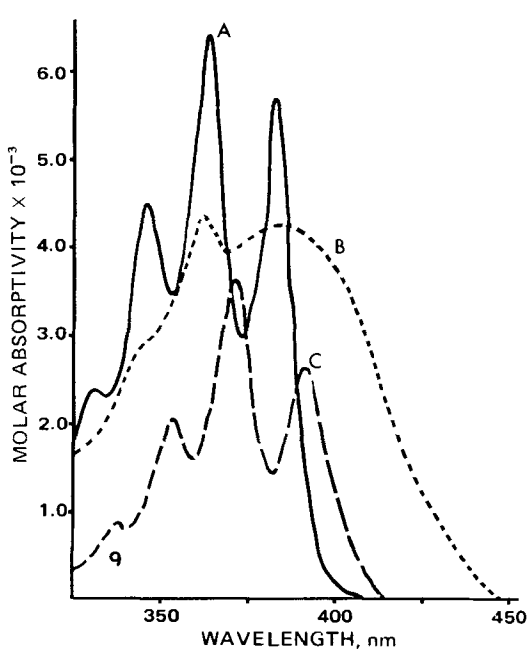


Figure 2—*Electronic absorption spectra of the spectroscopic probe II in aqueous solution (A), in concentrated sulfuric acid (B), and bound to DNA (C). All spectra were taken at 298 °K.*

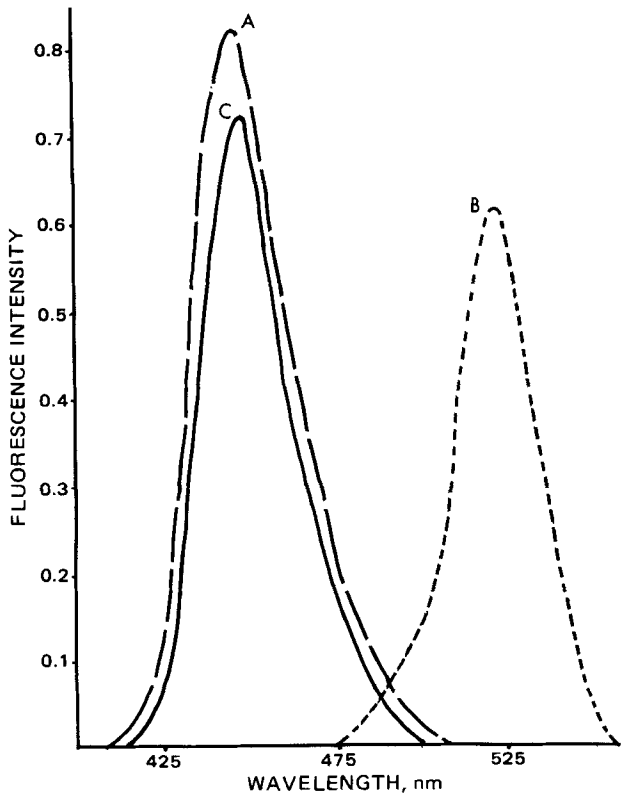


Figure 3—*Fluorescence spectra of the spectroscopic probe I in aqueous solution (A), in concentrated sulfuric acid (B), and bound to DNA (C). All spectra were taken at 298 °K.*

resonance energy attending conjugation may be sufficient to overcome the steric barrier to coplanarity, so that rotation of the 9-substituent into coplanarity with the ring may occur during the lifetime of the excited state.

The fluorescences from these compounds are usually diffuse and shifted to much longer wavelengths than in anthracene (17). Normally, the greater the positive charge on the exocyclic group in the 9-position, the greater is the tendency of electronic charge to be transferred to the exocyclic group and the smaller is the steric barrier to conjugation. Thus, in 9-anthroic acid and its cation, fluorescence is diffuse and lies at much longer wavelengths than in anthracene. However, in the 9-anthroate anion, the fluorescence is anthracene-like in appearance as a result of the failure of the carboxylate group to rotate into coplanarity with the anthracene ring during the lifetime of the ^1L_a state (17).

In amide II, the failure of the bulky substituent in the 9-position to conjugate in the ground state is evident from the highly structured, anthracene-like absorption spectrum of the unprotonated amide. Protonation of II results in a slight red shift of the long wavelength end of the absorption spectrum and some loss of vibrational structure. This result suggests better conjugation of the protonated exocyclic group with the anthracene ring. However, the small change in the absorption spectrum of II upon protonation, relative to that of I upon protonation, suggests that the exocyclic group of protonated II is not quite coplanar with the anthracene ring. The extremely small blue shift of the absorption spectrum of II going from water to heptane is reasonable in light of the low polarity of heptane and the lack of conjugation of the exocyclic group, which is the primary site of solvation, with the anthracene ring.

The blue shiftings of the fluorescence spectrum of II going from fluid water to rigid water or fluid heptane result from the same cause as previously described for I. The smallness of the shifts in this case is due to the fact that II is not well conjugated, even when fluorescence occurs in fluid solution. Therefore, the elimination of thermal relaxation by freezing or low solvent polarity is less pronounced than in the highly conjugated I. Protonation of II does result in a substantial red shift and loss of structure in the fluorescence spectrum, indicating some degree of conjugation between the exocyclic group and the anthracene ring. However, the shift in the fluorescence spectrum of II is much smaller than that occurring in I upon protonation. Even in the fluorescent state, the protonated form of II probably does not have its anthracene ring and its carboxamido group perfectly coplanar.

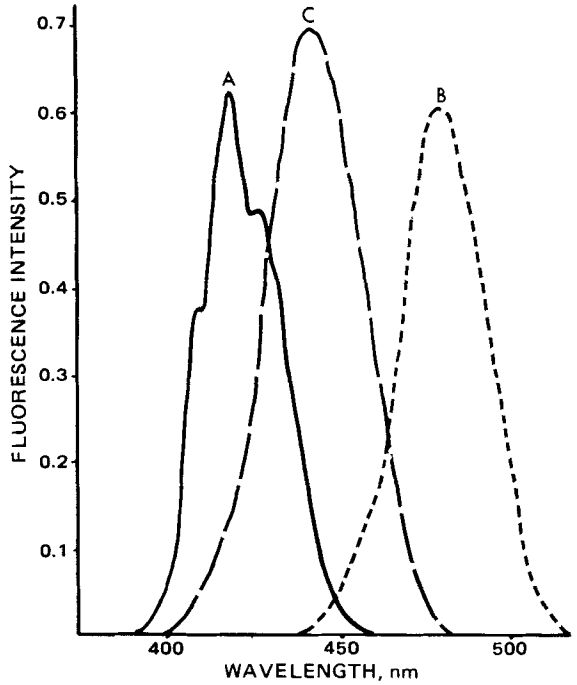


Figure 4—*Fluorescence spectra of the spectroscopic probe II in aqueous solution (A), in concentrated sulfuric acid (B), and bound to DNA (C). All spectra were taken at 298 °K.*

The binding of I and II to DNA results in a shifting of the absorption spectra to lower frequencies (longer wavelengths). In I, the long wavelength end of the absorption spectrum shifts slightly more in frequency than the short wavelength end (Table III), suggesting that the ^1L_b state is somewhat more affected than the ^1L_a state. In II, all vibrational features of the absorption spectrum are shifted in frequency by about the same amount. This result is unlike the spectroscopic effect resulting from transfer from water to a nonpolar solvent, which amounts to a slight blue shift of the absorption spectrum. Nor does it resemble the effect of protonation of the amido groups in which the absorption spectra red shift but also undergo dramatic changes in appearance.

Especially in II, where the exocyclic group is not conjugated with the anthracene ring in the ground state, a DNA-induced displacement of the absorption spectrum as large as that seen in Fig. 2 is not anticipated. It may, therefore, be concluded that the absorption spectral shifts produced by binding result primarily from the interaction of DNA with the π-electrons of the anthracene rings rather than with the carboxamido groups of I and II. If the anthracene rings of I and II are, in fact, intercalated between the base pairs of DNA, the interactions between the polymer and the aromatic portions of the probes could take several forms such as dative π-interaction with the electron-deficient purine and pyrimidine bases and dipole–dipole and dipole-induced dipole attraction. In addition, there would be mutual repulsion between the π-electrons of the anthracene rings and those of the purine and pyrimidine bases of DNA.

The red shifting of the absorption spectra of the probes, upon binding,

Table III—Frequency Shifts ($\Delta\bar{\nu}_a$) of the Discernible Vibronic Features of the Visible Absorption Spectra of I and II upon Binding to DNA in Water at pH 6.9

Without DNA		With DNA		
λ_a, nm	$\bar{\nu}_a$, $cm^{-1} \times 10^{-4}$	λ_a, nm	$\bar{\nu}_a$, $cm^{-1} \times 10^{-4}$	$\Delta\bar{\nu}_a$, $cm^{-1} \times 10^{-4}$
		I		
386	2.59	398	2.51	0.08
371	2.70	382	2.62	0.08
358	2.79	365	2.74	0.05
340	2.94	346	2.89	0.05
328	3.05	330	3.03	0.02
		II		
383	2.61	390	2.56	0.05
364	2.74	371	2.70	0.04
346	2.89	353	2.84	0.05
331	3.02	337	2.97	0.05
318	3.14	322	3.10	0.04

indicates that either the excited states of the bound probes are more stabilized than the ground states by attractive forces or that the ground states are more destabilized by repulsive forces in the bound probes than are the excited states. The fact that the long wavelength side (^1L_b) of the absorption spectrum of I shifts farther to the red than the short wavelength side (^1L_a) indicates that repulsive interaction between the π-systems is dominant in the spectrum. If I intercalates, with the carboxamido group sticking out into the surrounding solution (as it should if the cationic side chain is to be associated with the phosphate backbone of the DNA), the $^1L_b \leftarrow {}^1A$ transition would transfer electronic charge away from the intercalated anthracene ring to the carboxamido group.

The $^1L_a \leftarrow {}^1A$ transition would, however, occur entirely within the intercalated ring. In this case, there would be less interelectronic repulsion in the ^1L_b state than in the ^1L_a state, accounting for the greater red shift of the ^1L_b band as a result of binding to DNA. The observed greater red shift of the ^1L_b band in I cannot be explained in terms of the attractive forces between the anthracene ring and the purine and pyrimidine bases of DNA. With II, the entire *observed* absorption in the 300–400-nm region originates from the $^1L_a \leftarrow {}^1A$ transition (the ^1L_b band is weak and hidden under the ^1L_a band), so that the fact that all vibrational features of the absorption spectrum of II shift equally in frequency is not unexpected.

Since the fluorescence spectra taken in frozen media represent electronic transitions of the fluorophore in the equilibrium ground-state environment, it is not surprising that the DNA-induced fluorescence spectral shifts of I and II in rigid media are similar to those observed for the absorption spectra in fluid media. However, the fluorescence spectra taken in fluid solutions are quite different. The fluorescence spectrum of I when bound to DNA is unstructured and close in position in the electromagnetic spectrum to the fluorescence of free I in fluid solution. The fluorescence of II when bound to DNA in fluid solution has lost its vibrational structure and is shifted to longer wavelengths. This phenomenon strongly suggests that, subsequent to excitation, the anthracene ring and the substituted carboxamido group in the 9-position have moved closer to coplanarity than in the ground state. Both of these observations are indicative of thermal relaxation in the time between absorption and emission.

In I, where the conjugation of the exocyclic group and the aromatic ring is appreciable even in the ground state, thermal relaxation in the excited state is primarily derived from the reorientation of the solvent cage around the carboxamido group. In II, the predominant effect is that the anthracene ring and the 9-carboxamido function come into better conjugation subsequent to absorption and prior to fluorescence. Solvent reorientation alone would not produce much of a change in the fluorescence spectrum of II if the carboxamido group and the anthracene ring were perpendicular (or nearly so) with respect to one another. This means that intercalated II has some freedom of rotation about the bond joining the anthracene ring to the carboxamido group.

It is interesting that II does not become conjugated in the excited state in heptane or water but does when bound to DNA. In a previous study (18), 9-anthracenecarboxamide, a more basic, less hindered precursor of II that does not bind to DNA, became conjugated in the excited state in aprotic solvents but not in water due to steric interference by the aqueous solvent cage around the carboxamido group. The failure of II to become conjugated in the excited state in water or heptane is likely due primarily to the steric restrictions brought about by the bulky side chain substituted onto the basic anthracene structure and the short lifetime of the excited probe. However, the lifetime of the lowest excited singlet state of II increases about sevenfold and its fluorescence efficiency increases about fivefold as a result of binding (Table II).

The reciprocal of the lifetime of the lowest excited singlet state is the sum of the unimolecular rate constants for fluorescence, internal conversion, and singlet–triplet intersystem crossing. For II in fluid aqueous solution, this quantity is 3.3×10^8 sec^{-1}; for II bound to DNA, it is 4.65×10^7 sec^{-1}. Moreover, the ratio of the quantum yield of fluorescence to the lifetime of the lowest excited singlet state is the rate constant for fluorescence alone (*i.e.*, the rate constant for deactivation if fluorescence is the only process deactivating the lowest excited singlet state). For II in fluid aqueous solution, the rate constant for fluorescence is 1.0×10^7 sec^{-1}; for II bound to DNA, it is 6.5×10^6 sec^{-1}.

The sum of the rate constants for radiationless deactivation of II in fluid aqueous solution and bound to DNA are 3.2×10^8 and 4.0×10^7 sec^{-1}, respectively. Since no phosphorescence was observed for any of the species at 77 °K, it may be assumed that radiationless deactivation is mainly limited to internal conversion, *i.e.*, radiationless deactivation due to molecular motion. Since the rate of internal conversion is, in this case, the dominant factor governing the lifetime of the excited state, it is reasonable to surmise that the slower nonradiative deactivation of the singlet state of II resulting from binding to DNA is the result of the decreased freedom of molecular motion. Probably this entails minimization of the random motion of the alkyl side chain by "pinning" to the DNA phosphate backbone and the entrapment of the anthracene ring of II in the helix. The greater lifetime and the restricted motion of the side chain of photoexcited bound II provide a satisfactory explanation for the occurrence of excited-state conjugation in the DNA-bound probe.

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