v_r = velocity of flow in the r direction (cylindrical coordinates)
V_{spread}, V_{visco} = average velocity with which the finger moves in assessment of spreadability and viscosity
μ = viscosity of Newtonian fluids
τ_{zx}, τ_{zr} = components of shear stress
τ_{spread}, τ_{visco} = stress exerted by the fluid on the finger during assessment of spreadability and apparent viscosity, respectively

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Electronic Spectra and Electronic Structures of Some Antimicrobials Derived from Proflavine

STEPHEN G. SCHULMAN [x], DATTA V. NAIK, ANTHONY C. CAPOMACCHIA, and TIMOTHY ROY

Abstract □ The shifts in the absorption and fluorescence spectra of 3-aminoacridine, proflavine, acridine orange, and acridine yellow were employed to show that the singly charged cations, the predominant species at biological pH, exist in the ground state in the amino form. In the lowest excited singlet state, however, the monocations of the diaminoacridines have the imino structure, a conclusion supported by the relative ground- and excited-state pKa values of the reactions of the monocation with H^+. The ground-state amino structure has its positive charge concentrated at the heterocyclic nitrogen atom, a fact that is of primary importance in determining the geometry of binding to DNA.

Keyphrases □ 3,6-Diaminoacridine derivatives—electronic spectra and electronic structures □ Proflavine derivatives—electronic spectra and electronic structures □ Antimicrobials—3,6-diaminoacridine derivatives, electronic spectra and electronic structures □ Electronic spectra and structure—diaminoacridine dyes, dependence upon solvent properties and state of protonation □ DNA—interaction with diaminoacridine dyes, electronic spectra and structure of dyes

Several dyes (acridine orange and acridine yellow) derived from proflavine (3,6-diaminoacridine) are employed as bacteriostatic agents, biological stains, and spectroscopic probes of the interactions of nucleic acids with small molecules (1). These substances are well known for their mutagenic effects arising from their interactions with nucleic acids (2). The singly charged cations, which are the prevalent prototropic species, at biological pH derived from proflavine (3,6-diaminoacridine), acridine orange [3,6-bis(dimethylamino)acridine], and acridine yellow (2,7-dimethyl-3,6-diaminoacridine) have been employed extensively in binding studies with nucleic acids (3). These dyes exhibit electronic absorption and fluorescence spectral changes upon binding to DNA.

The interactions of the diaminoacridines with DNA are generally believed to entail electrostatic association of the site of positive charge, in the singly protonated dye molecule, with a negatively charged phosphate ester linkage of the nucleic acid "backbone." In the case of proflavine, it has been shown that the remainder of the dye molecule is inserted between two base pairs of the double helix (or between individual bases in single-stranded nucleic acids), oriented toward the center of the helix and stabilized by hydrophobic interaction with the helix (2). There is, however, some disagreement as to whether the positive charge of the singly protonated diaminoacridine cations is predominately concentrated at the heterocyclic nitrogen atom of the acridine ring (the site of protonation) or at one of the amino nitrogen atoms. This argument arises from the possibility of writing the valence bond structures of the singly protonated cations of proflavine in either of two ways (I and II).

Table I—Long Wavelength Absorption (λ_{1L_a} and λ_{1L_b}) and Fluorescence (λ_f) Maxima of 3-Aminoacridine and Several 3,6-Diaminoacridines in Various States of Protonation in Aqueous and Sulfuric Acid Media

Compound	Species[a]	λ_{1L_b}, nm	λ_{1L_a}, nm	λ_f, nm
3-Aminoacridine	D	353	402	480
	M	365	452	528
	N	353	408	522
3,6-Diaminoacridine (proflavine)	T	348	395	477
	D	361	458	535
	M	—	443	504
	N	—	393	514
3,6-Bis(dimethylamino)acridine (acridine orange)	T	351	395	480
	D	370	494	575
	M	—	490, 477	530
	N	—	435	535
2,7-Dimethyl-3,6-diaminoacridine (acridine yellow)	T	360	408	489
	D	372	460	532
	M	—	444	502
	N	—	394	498

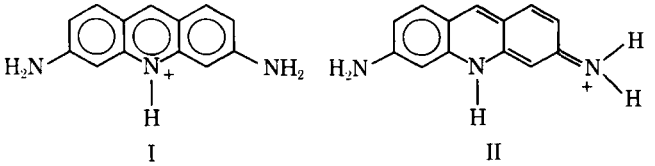
[a] T = triply charged cation, D = dication, M = monocation, and N = neutral species.

Consequently, two models for the geometry of the intercalative binding of proflavine-like dyes have been proposed: one with the heterocyclic nitrogen atom directed toward a phosphate ester group, leaving both exocyclic amino groups outside the helix, and one with an exocyclic nitrogen atom directed toward a phosphate ester group, having the other exocyclic group directed very nearly toward the center of the helix (3, 4). Löber (5) noted the similarity between the absorption and fluorescence spectra of singly protonated acridine orange and pyronine G, the xanthene analog of acridine orange, and suggested this similarity as evidence for the latter intercalation model. In pyronine G, the unprotonated molecule is singly charged and, because of the greater electronegativity of oxygen relative to nitrogen, Structure III is favored over Structure IV. Consequently, Structure V would, by spectral analogy, be favored over Structure VI for singly protonated acridine orange.

To elucidate the electronic structures of the diaminoacridine dyes and, ultimately, their interactions with DNA, the present study of the dependence of their electronic spectra upon solvent properties and state of protonation was undertaken.

EXPERIMENTAL

The pH measurements were made on a pH meter[1] with a combination silver–silver chloride glass electrode. Electronic absorption spectra were taken in 1-cm silica cells on a grating-type spectrophotometer[2]. Fluorescence measurements were taken on a fluorescence spectrophotometer[3] whose monochromators were calibrated against the line emission spectrum of xenon. Emission spectra were corrected for the wavelength response of the monochromators and phototube by means of a corrected spectra accessory employing a rhodamine-B quantum counter.



I

II

[1] Model 801, Orion Research, Inc., Cambridge, Mass.
[2] Model DB-GT, Beckman Instruments, Inc., Fullerton, Calif.
[3] Model MPF-2A, Perkin-Elmer Corp., Norwalk, Conn.

Table II—Long Wavelength Absorption (λ_{1L_a} and $\lambda^1_{L_b}$) and Fluorescence (λ_f) Maxima of 3-Aminoacridine and Several 3,6-Diaminoacridines in Various States of Protonation in Chloroform

Compound	Species[a]	λ_{1L_b}, nm	λ_{1L_a}, nm	λ_f, nm
3-Aminoacridine	M	368	462	530
	N	352	407	495
Proflavine	M	—	445	505
	N	—	397	497
Acridine orange	M	—	492	518
	N	—	432	498
Acridine yellow	M	—	446	503
	N	—	397	489

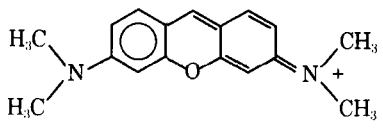
[a] M = monocation, and N = neutral species.

3-Aminoacridine was prepared by the deamination of proflavine according to the method of Martin and Tong (6). Proflavine[4], acridine orange[4], and acridine yellow[4] were purified by several recrystallizations of each from absolute ethanol.

Absorption and fluorescence spectra were taken on 1×10^{-5} *M* solutions of 3-aminoacridine, proflavine, and acridine yellow. However, because of the aggregation of acridine orange, which is appreciable at $\sim 10^{-5}$ *M*, the spectra of this compound were taken on 1×10^{-6} *M* solutions. The spectrophotometric titration procedures employed were described previously (7).

RESULTS

The electronic spectral maxima of 3-aminoacridine and of the diaminoacridines in various states of protonation, in aqueous and sulfuric acid media, are presented in Table I. The spectra of 3-aminoacridine are shown in Fig. 1. The spectral maxima of the neutral species and monocation derived from each compound, in chloroform, are presented in Table II. The pKa values (determined absorptiometrically) for the various prototropic reactions are listed in Table II. Also presented in Table II are the inflection points (pKa*) in the fluorometric titration curves corresponding to each measurable excited-state reaction.



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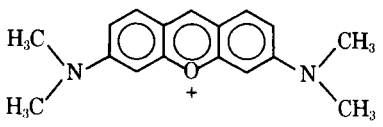

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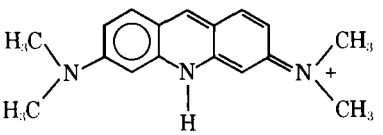
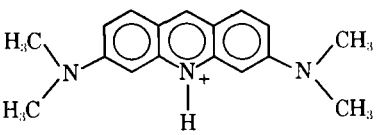


V



VI

[4] Matheson, Coleman and Bell, East Rutherford, N.J.

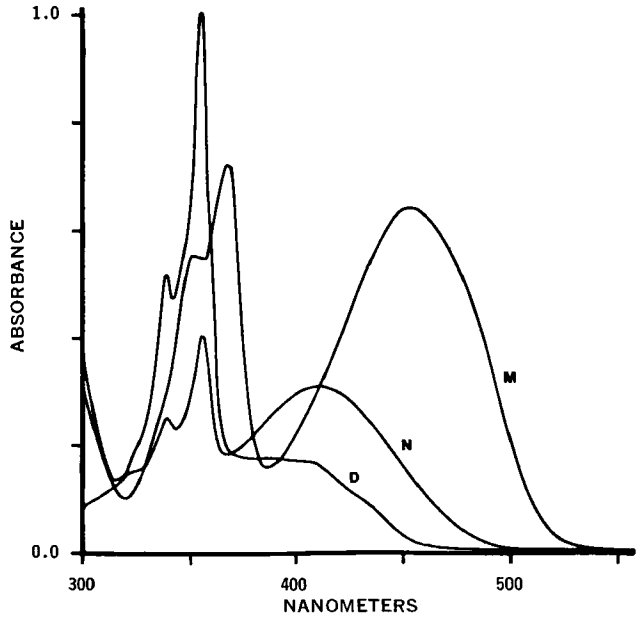


Figure 1—*Absorption spectra of 3-aminoacridine* ($\sim 5 \times 10^{-5}$ M). *Key: N, neutral; M, monocation; and D, dication.*

In each compound studied, only the neutral species ⇌ monocation interchange did not measurably occur during the lifetime of the excited state. The pKa* values for the trication ⇌ dication and dication ⇌ monocation excited-state reactions are believed to correspond very closely to the excited-state dissociation constants for these reactions.

DISCUSSION

The longest wavelength absorption bands of arylamines may be thought to originate from the transfer of electronic charge from the amino group to the vacant π^*-orbitals of the aromatic ring as a result of the interaction of the ground-state molecular electronic distribution with the electric vector of the exciting light (8). In the ground state, the aromatic ring is weakly conjugated with the amino group; but in the excited state the amino group is more extensively conjugated with the aromatic ring, resulting in stabilization of the excited state relative to the ground state. As a result, the longest wavelength absorption band of the aminoaromatic lies at a lower energy or longer wavelength than the longest wavelength absorption band of the parent hydrocarbon.

Protonation of the amino group removes the lone pair from conjugation with the aromatic ring in ground and excited states, resulting in an absorption spectrum very similar to that of the parent aromatic hydrocarbon but slightly displaced due to the polarizing influence of the protonated amino group. The second longest wavelength band of an arylamine originates from an essentially ring-localized transition and is relatively unaffected by protonation of the amino group (8). The fluorescence of an arylamine, which entails the spontaneous transfer of charge from the aromatic ring back to the amino group, lies at longer wavelengths than that of the parent hydrocarbon. However, the excited state from which fluorescence occurs and the ground state to which it occurs are somewhat different than the corresponding states involved in the longest wavelength absorption process.

The weakly conjugated amino group in the thermally equilibrated ground-state molecule is essentially tetrahedral in geometry. Because electronic transitions are somewhat faster than nuclear motions, the amino group in the electronically excited state, produced by absorption (the Franck–Condon excited state), is still predominately tetrahedral. However, subsequent to light absorption and prior to fluorescence, the amino group becomes planar (or nearly so) with respect to the aromatic ring to give the molecular configuration of lowest energy in accord with the highly conjugated excited amine. When fluorescence occurs, the light emission process is so rapid that the ground state in which the molecule initially arrives is thermally excited, retaining the planar amino group of the excited-state equilibrium geometry. Because the excited state involved in fluorescence is lower in energy than that involved in absorption and the ground state involved in fluorescence is higher than that involved in absorption, the fluorescence lies at longer wavelength than the longest wavelength absorption band. It can be concluded that, although both the absorption and fluorescence processes in question involve transition between the ground and first excited singlet states, the absorption process reflects the ground-state molecular structure while the fluorescence process reflects the excited-state atomic configuration.

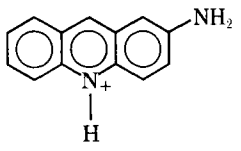
In nitrogen heterocycles such as acridine, the introduction of amino groups produces spectral effects similar to those just discussed but with some qualitative differences. The nitrogen atom is more electronegative than carbon and, therefore, exerts an attractive influence on the π-electron system of the aromatic ring as well as on the lone pair of an amino substituent (9). Because the excited states of aromatic rings and arylamines are generally more polar than their ground states, the attractive or stabilizing influence of the nitrogen atom is generally greater upon the excited state than upon the ground state. The result is that the longest wavelength absorption and fluorescence bands of *N*-heterocyclic amines lie at longer wavelengths than in the corresponding carbocyclic systems. This effect is much more pronounced in ring-protonated *N*-heterocyclic amines, in which the ring-protonated nitrogen is even more electronegative.

In aminoacridines, the longest wavelength absorption and fluorescence bands [the ^1L_a bands in the Klevens and Platt (10) nomenclature system] are extremely sensitive in position to the state of protonation of the heterocyclic nitrogen atom. The second absorption band (^1L_b) of an aminoacridine, however, is much less sensitive to protonation of the heterocyclic nitrogen atom and is indicative of the acridine ring system, lying at ~350–370 nm in monosubstituted acridines.

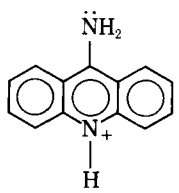
At this point, it is desirable to distinguish between two types of ring-protonated amino *N*-heterocycles: Type 1, those for which conjugation of the amino group with the aromatic ring cannot be represented by low energy canonical structures, *e.g.*, 2-aminoacridinium ion (VII); and Type 2, those for which conjugation of the amino group with the aromatic ring can be depicted by a formal low energy valence-bond representation, *e.g.*, 9-aminoacridinium ion (VIII and IX).

Protonated *N*-heterocyclic amines of the first type are weakly conjugated in the ground state because of orbital occupancy restrictions. However, they are substantially conjugated in the lowest excited state because the π^*-orbitals, which become occupied in the excited state, provide an electronic pathway between the amino group and the aromatic ring (7). Therefore, these compounds are characterized by spectroscopic behavior of the kind discussed up to this point. Moreover, the dissociation constants of these species are typical of the class, having pKa ~5–6 and pKa ~0–1 for dissociation from the ring nitrogen atom and for protonation of the amino group, respectively (7).

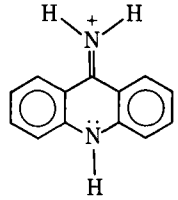
The second type of protonated *N*-heterocyclic amines may be extensively conjugated in the ground state because the canonical structure alternative to the protonated *N*-heterocyclic amine (the iminium ion) provides a low energy electronic distribution, which does not violate the restrictions imposed by the Pauli exclusion principle (7). The iminium structure may be regarded as the ex-



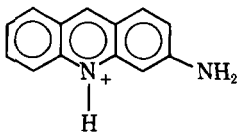
VII



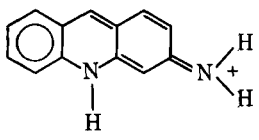
VIII



IX



X



XI

treme limit of conjugation of the amino group with the protonated heterocyclic ring. However, in such a structure, it is clearly impossible for light absorption to transfer charge from the exocyclic group to the ring. Rather, because of the positive charge on the exocyclic group, the low energy π^*-orbital associated with this group (similar to a carbonyl group), and the lone pair localized on the heterocyclic nitrogen atom, absorption entails transfer of charge from the ring to the exocyclic group so that conjugation of the exocyclic group is actually less extensive in the lowest excited singlet state than in the ground state.

In this extreme situation, the ground state would be relatively more stabilized by the presence of the amino group and, as in the case of the 9-aminoacridinium ion, the ^1L_a absorption and fluorescence spectra would be expected to lie at shorter wavelengths than those of the acridinium cation (7). Moreover, the loss of aromaticity in the ground state should, as in the case of the 9-aminoacridinium monocation, shift the ^1L_b band (characteristic of aromatic ring size) to shorter wavelengths.

Dissociation or protonation of the monocation restores the aminoacridine or ammonium acridine structure, and either process thus shifts the spectra to longer wavelengths. The pKa values of these compounds also reflect the loss of positive charge at the ring nitrogen atom and the gain of positive charge at the exocyclic nitrogen atom in the ground state. For example, in 9-aminoacridinium monocation, pKa = 10.0 for the heterocyclic nitrogen atom and −8.5 (7) for the exocyclic nitrogen atom. Moreover, the dissociation constants of the excited species of the Type I ring-protonated heterocyclic amines are more basic at the ring nitrogen atom and less basic at the exocyclic nitrogen atom while those of the Type 2 compounds are in the opposite order, in accord with the opposite directions of flow of electronic charge in either type of ring-protonated heterocyclic amine (7).

As previously mentioned, the 9-aminoacridinium ion and the 2-aminoacridinium ion represent two extremes of conjugative behavior in ring-protonated *N*-heterocyclic arylamines. In the 9-aminoacridinium cation, the 9-iminoacridan cation form predominates because it is not only allowed electronically but also is electrostatically favored by the proximity between the heterocyclic and exocyclic nitrogen atoms as well as by the inherently low aromatic stability of the central ring of anthracene and acridine derivatives. However, in certain ring-protonated aminoacridines, such as 3-aminoacridine, the amino group is considerably more separated from the heterocyclic function so that the polarization of the amino lone pair of X to form XI is a weaker interaction than in the 9-aminoacridinium cation.

Moreover, the formation of XI from X entails the disruption of two aromatic rings, including one that is normally stabilized by an aromatic sextet, and is, therefore, less favorable electronically than the corresponding process in the 9-aminoacridinium cation. Under such circumstances, it is to be expected that the electronic distribution in the 3-aminoacridinium ion would be intermediate between Structures X and XI. In this case, the long wavelength electronic absorption band of the 3-aminoacridinium ion could entail transfer of electronic charge away from the amino group and toward the heterocyclic function if the actual structure is closer to the amino form (X), in the ground state, or transfer of electronic charge toward the imino group and away from the heterocyclic function if the structure is closer to that of the imino form (XI). If X more accurately represents the structure of the 3-aminoacridinium monocation, the long wavelength absorption band should shift to shorter wavelengths upon dissociation from the heterocyclic group or protonation of the amino group while the corresponding prototropic processes should result in absorption spectral shifts to longer wavelengths if XI is closest to the actual structure.

Similar arguments are applicable to the shifting of the fluorescence spectra of the 3-aminoacridinium monocation upon protonation or dissociation. However, because of the conformational relaxation that takes place in the excited state subsequent to absorption and prior to emission, it is possible that the electronic charge

Table III—Dissociation Constants for the Prototropic Equilibria in the Ground Electronic State (pKa) and in the Lowest Excited Singlet State (pKa*) (Where Measurable by Fluorometric pH Titrimetry) of 3-Aminoacridine and of the 3,6-Diaminoacridines[a]

Compound	$T \rightleftharpoons D + H^+$	$D \rightleftharpoons M + H^+$	$M \rightleftharpoons N + H^+$
3-Aminoacridine			
pKa	—	−1.4	8.0
pKa*	—	−5.6	
Proflavine			
pKa	−2.7	0.3	9.5
pKa*	−9.0	1.6	
Acridine orange			
pKa	−3.3	0.2	10.1
pKa*	−7.6	1.1	
Acridine yellow			
pKa	−3.0	0.5	8.9
pKa*	−7.3	1.5	

[a] T = triply charged cation, D = dication, M = monocation, and N = neutral species.

redistribution produced by going from the ground state to the lowest excited singlet state would convert the predominately amino-type ground-state monocation X into the predominately imino-type excited-state monocation (XI) or vice versa. The result of such an interaction would be that protonation and dissociation of the monocation would produce absorption and fluorescence shifts in opposite directions.

In 3-aminoacridine, the shifts of the ^1L_a absorption and fluorescence bands of the monocation upon dissociation to the neutral species or protonation to the dication, the presence of a well-defined ^1L_b band (between 350 and 370 nm) in all species, and the greater acidity of the dication in the lowest excited singlet state relative to the ground state all indicate that the monocation of 3-aminoacridine is predominately aminoacridine-like in ground and lowest excited singlet states (X).

In proflavine, the presence of the second amino group in the monocation results in a somewhat more complicated picture. The triply and doubly charged cations derived from proflavine are similar in electronic structure to the dication and monocation, respectively, derived from 3-aminoacridine. Because of the absence of lone pairs on both amino groups, the spectra of the trication of proflavine are similar to those of an unsubstituted acridinium ion (the iminium structure is impossible in the trication). Dissociation of one amino group to form the dication frees one lone pair for the interaction with the aromatic system. Because of the positive influence of the remaining protonated amino group, the charge-transfer interaction between the free amino group and the heterocyclic ring is enhanced, with the greatest stabilization effected on the excited state because of its great charge-transfer character. As a result, the ^1L_a and fluorescence bands of the dication lie at slightly longer wavelengths than those of the monocation of 3-aminoacridine (because of the lack of the $-NH_3^+$ group in the latter) as well as at much longer wavelengths than the corresponding spectra of the trication.

This behavior is anticipated for the spectrum of the amino form of the dication and is supported by the occurrence of the ^1L_b band typical of an acridine ring as well as by the fact that the free amino group is less basic in the first excited singlet state than in the ground state. If the imino form predominated, the loss of aromaticity in the acridine ring should have shifted the ^1L_b, ^1L_a, and fluorescence bands to shorter wavelengths while the imino structure of the exocyclic group should have resulted in enhanced basicity in the lowest excited singlet state.

In the monocation derived from proflavine, the ^1L_a absorption and fluorescence bands lie at wavelengths shorter than those of the corresponding spectral bands in both the monocation of 3-aminoacridine and the dication of proflavine. Moreover, the ^1L_b band in the proflavine monocation is not visible. The lack of appearance of the ^1L_b band is not in itself indicative, because this band could be extremely weak in intensity and buried under the tailing of the extremely intense ^1L_a band in the monocation of proflavine. However, the shifting of the ^1L_a absorption and fluorescence bands and the fact that pKa* > pKa for the dication–monocation intercon-

version suggest that at least the excited monocation is in the imino form (II).

Upon dissociation of the monocation of proflavine (from the heterocyclic nitrogen atom) to form the neutral species, the ^1L_a absorption band shifts to a shorter wavelength by an amount comparable to the corresponding shift in 3-aminoacridine. However, the fluorescence band of the monocation moves to a slightly longer wavelength upon dissociation from the heterocyclic nitrogen atom. These results indicate that, in the ground state, the monocation is predominately in the amino form (as 3-aminoacridine) and support the conclusion that in the lowest excited singlet state the monocation fluoresces as the imino valence tautomer. The neutral molecule is, of course, amine-like in ground and excited states, since an imino form in this species would be an inherently unstable zwitterion with positive and negative charges both borne by nitrogen atoms (*i.e.*, atoms of comparable electronegativity).

The small shift of the ^1L_a band of the dication to shorter wavelengths upon dissociation to the monocation can be rationalized in terms of the ground-state amino structures of both the dication and monocation as follows. Since the ^1L_a band arises from the promotion of an amino group lone pair electron to a π^*-orbital of the acridinium ring, the release of an additional lone pair of comparable energy, by dissociating the dication to the monocation, does not have much effect upon the orbital aspect of the transition. However, while the positive charge of the ammonium group of the dication enhances charge transfer in the excited state and thereby lowers the energy of the transition relative to that in the 3-aminoacridine monocation, the extra lone pair in the proflavine monocation causes greater electron repulsion in the charge-transfer excited state and thereby destabilizes the excited state with respect to the ground state. Hence, the ^1L_a band of the proflavine monocation lies at a shorter wavelength than that of the proflavine dication or the monocation of 3-aminoacridine, while the ^1L_a band of the neutral proflavine lies at a shorter wavelength than that of neutral 3-aminoacridine.

The spectroscopic and prototropic behavior of acridine orange is comparable to that of proflavine. However, the 2,7-dimethyl-substituted proflavine, acridine yellow, behaves differently in one way. The fluorescence of the neutral molecule is at slightly shorter wavelength than that of the monocation. However, the excited-state pKa* of the dication–monocation interconversion is more basic than the corresponding ground-state pKa, indicating that the imino structure of the monocation is in the excited state. The anomalous fluorescence shift observed upon going from the monocation to the neutral molecule in acridine yellow appears to be due to greater steric inhibition, by the methyl groups in the 2- and 7-positions, of conjugation in the excited state of the neutral species, resulting in an anomalously short fluorescence wavelength in this species. The apparently greater sensitivity to solvent polarity or hydrogen-bonding capacity of the fluorescences of the neutral proflavine and acridine orange molecules relative to that of the corresponding monocations also reverses the order of the fluorescence maxima of proflavine and acridine orange, although the amino and imino structures of the neutral molecules and monocations are, presumably, preserved in chloroform (Table II).

In light of the results of these experiments, it may be predicted that proflavine, acridine orange, and acridine yellow bind in their monocation forms to DNA in such a way that the positively charged heterocyclic nitrogen atom is oriented toward a phosphate ester group. However, upon excitation, the sites of predominant positive charge in the monocations shift to the exocyclic nitrogen atoms. Thus, the excited dye molecules bound to DNA will generally not be in the binding configuration of lowest electrostatic energy. They will tend to reorient themselves as best they can, before fluorescing, to achieve the lowest energy configuration. Although translational motions of the bound entities are severely restricted, the lifetime of a typical fluorescent excited state is more than adequate to allow for rotational and vibrational rearrangements. Consequently, electronic absorption spectroscopy appears to be a very powerful and readily available tool for the probing of drug–receptor interactions. However, much more work remains to be done before fluorescence spectroscopy can enjoy the same interpretive versatility.

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