Valence Tautomerism of Singly Protonated 9-Aminoacridine and Its Implications for Intercalative Interactions with Nucleic Acids

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Abstract □ Electronic absorption and fluorescence spectroscopy were used to show that the singly protonated 9-aminoacridine cation exists as the vinylogous cyclic amidine valence tautomer, the 9-iminoacridan monocation, in ground and excited states when in aqueous solution at physiological pH. The evidence indicates that the 9-aminoacridine monocation may intercalate into double-helical DNA in an orientation different from that employed by other acridine derivatives.

Keyphrases □ 9-Aminoacridine—valence tautomerism of singly protonated form, implications for intercalative interactions with nucleic acids, electronic absorption and fluorescence spectroscopy □ Acridine derivatives—valence tautomerism of singly protonated 9-aminoacridine, implications for intercalative interactions with nucleic acids, electronic absorption and fluorescence spectroscopy □ Nucleic acids—intercalative interactions, valence tautomerism of singly protonated 9-aminoacridine □ 9-Iminoacridan monocation—tautomer form of singly protonated 9-aminoacridine cation, intercalation with double-helical DNA

Aminoacridines are used extensively in the medical sciences as research tools and to a lesser extent clinically. 9-Aminoacridine has been used primarily as a probe to study the binding of small molecules to biologically important macromolecules. The binding of aminoacridines by intercalation into double-helical and denatured DNA and single-strand RNA has been extensively studied (1–5). Albert (6) demonstrated that the aminoacridine cation is necessary for bacteriostasis. The implication is that the singly protonated form is necessary for intercalation to occur. To date, the position and orientation of the 9-aminoacridine monocation during intercalation are undetermined. Studies have suggested that it may intercalate with the protonated heterocyclic nitrogen atom oriented toward the negatively charged phosphate ester linkages of double-helical DNA. However, it has recently been shown that 2- and 4-aminoquinolines exist as cyclic amidines rather than heterocyclic amines, having most of their positive charge at the exocyclic nitrogen atom when singly protonated (7). This implies that 9-aminoacridine, a benzolog of 4-aminoquinoline, may also exist as the cyclic amidine (I) rather than as the amino form (II) when singly protonated. Zanker and Wittwer (8) suggested the possibility of this structure as a resonance form of the monocation.

If the 9-aminoacridine monocation does exist as the cyclic amidine, its position when intercalated may differ from that of other aminoacridines due to residence of the positive charge of protonation on the exocyclic nitrogen.

The present study was undertaken to determine whether the singly protonated 9-aminoacridine cation exists as the amine or the cyclic amidine in aqueous solutions at physiological pH. Comparisons of electronic spectral shifts induced through changes in the state of protonation or solvent environment as well as dissociation constants of 9-aminoacridine and 2-aminoacridine (the latter was assumed to represent "ordinary" aminoacridines) were used to show that 9-aminoacridine behaves differently from arylamines and heterocyclic arylamines in particular.

EXPERIMENTAL

The pH measurements were made on a pH meter[1] with a combination silver–silver chloride glass electrode[2]. 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 monochromators and the phototube by means of a corrected spectra accessory[3] employing a rhodamine-B quantum counter.

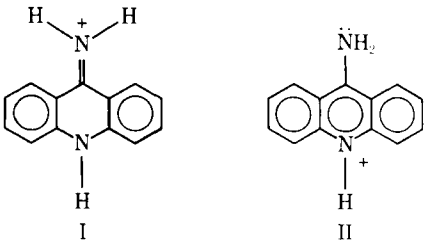
2-Aminoacridine[4], 9-aminoacridine[5], and 9-aminoacridine[6] monohydrochloride were purified by multiple recrystallizations from ethanol.

Reagent grade sulfuric acid[7] and spectroquality chloroform[8], *n*-heptane[8], and dioxane[8] were used as solvents. Benzene-free ethanol[8] and distilled, deionized water were used as necessary. Sodium hydroxide[7] USP was used to make an 18 *M* solution.

Ethanolic or aqueous solutions (about 1×10^{-3}) of the compounds to be studied were delivered from a 100-μl micropipet[9] into 10-ml volumetric flasks containing aqueous solutions at the desired pH. The added 0.1 ml caused negligible volume change. The ethanolic solutions employed were too dilute to affect pH. All solutions were prepared immediately before use to eliminate decomposition errors and errors due to pH changes of poorly buffered solutions in the mid-pH range.

RESULTS

Table I contains the long wavelength absorption and fluorescence band maxima for the prototropic species derived from 9-aminoacridine and 2-aminoacridine. The longest wavelength vibrational features of the absorption bands and the shortest wave-



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[2] Beckman Instruments, Inc., Fullerton, Calif.
[3] Perkin-Elmer Corp., Norwalk, Conn.
[4] Dr. D. Jackman, Texas Technical University, Lubbock, Tex.
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Table I—Electronic Absorption (λ_a) and Fluorescence (λ_f) Maxima of the Doubly Protonated Cations (*D*), Singly Protonated Cations (M), Neutral Molecules (N), and Anions (A) Derived from 2-Aminoacridine and 9-Aminoacridine in Aqueous and Sulfuric Acid Media[a]

	D		M		N		A	
	λ_a, nm	λ_f, nm	λ_a, nm	λ_f, nm	λ_a, nm	λ_f, nm	λ_a, nm	λ_f, nm
2-Aminoacridine:								
O–O band	425	—	—	—	—	—	—	—
Maximum	402	475	460	592	405	530	—	—
9-Aminoacridine:								
O–O band	440	475	423	430	426	445	443	470
Maximum	418	496	402	455	406	470	422	497

[a] The spectra of D were taken in solutions of 18 *M* H_2SO_4(H_0-10), the spectra of M were taken in water at pH 4.0, the spectra of N were taken in dilute sodium hydroxide in water (pH 11.5), and the spectra of A were taken in 14.2 *M* aqueous sodium hydroxide (H- 17.5).

length vibrational features of the fluorescence bands are taken as the O–O bands of absorption and fluorescence, respectively, for all compounds studied which demonstrated vibrational structure in their electronic spectra. The electronic absorption and fluorescence spectra of all species (anion, neutral, monocation, and dication) obtained from 9-aminoacridine (Figs. 1 and 2) exhibit well-defined vibrational features. The electronic absorption and fluorescence spectra of all species obtained from 2-aminoacridine (Figs. 3 and 4) exhibit diffuse spectral bands with the position of the O–O bands being uncertain, except the absorption spectrum of the dication which is structured.

The ground-state dissociation constants of 9-aminoacridine and 2-aminoacridine were determined by absorptiometric pH titrimetry and are presented in Table II. Also listed in Table II are the excited-state dissociation constants (pKa*) of 9-aminoacridine and 2-aminoacridine determined by fluorometric pH titrimetry. Excited-state dissociation constants were not obtained for the first protonation of 2-aminoacridine and 9-aminoacridine because the variations of the fluorescence quantum yields of the neutral species and monocations with pH followed ground-state titration characteristics. Apparently, the rates of proton exchange between these excited species are much slower than their rates of radiative deactivation.

A study of the spectra of 2-aminoacridine and 9-aminoacridine and their monocations in solvents of varying hydrogen-bonding capability and polarity produced the data in Table III. The electronic absorption and fluorescence spectra of the 9-aminoacridine monocation were barely affected by a change of solvent, showing a slight trend toward shorter wavelengths as solvent polarity was increased. In contrast, 2-aminoacridine and its monocation demonstrated trends toward substantially longer wavelengths in both their electronic absorption and fluorescence spectra as solvent polarity was increased.

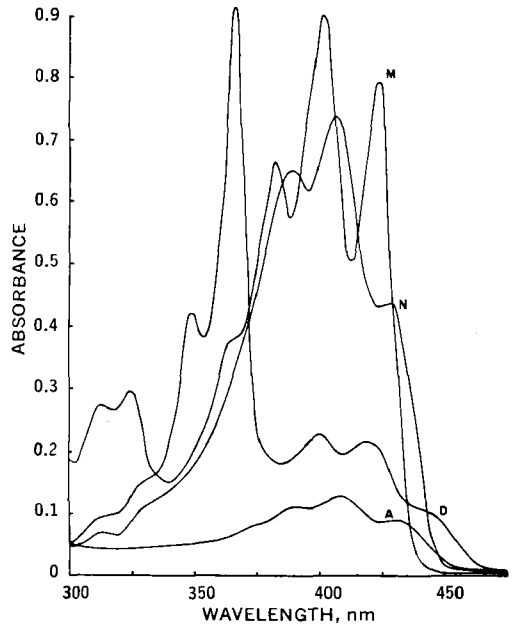


Figure 1—*Electronic absorption spectra of the dication (D), monocation (M), neutral species (N), and anion (A) derived from 9-aminoacridine at room temperature.*

DISCUSSION

2-Aminoacridine exemplifies the spectroscopic and prototropic behavior of a "well-behaved" *N*-heterocyclic arylamine (9–12). The neutral molecule has absorption and fluorescence maxima at longer wavelengths than those of acridine (λ_a = 362 nm, λ_f = 425 nm), the parent heterocyclic nucleus, while the singly protonated cation absorbs and fluoresces at wavelengths considerably longer than those of its parent heterocyclic nucleus, the acridinium cation (λ_a = 405 nm, λ_f = 481 nm). The doubly charged cation derived from 2-aminoacridine absorbs and fluoresces at wavelengths close to the corresponding spectral maxima of the acridinium cation. The pKa for the ground-state equilibrium between the neutral molecule and the singly charged cation of 2-aminoacridine is only slightly less acidic than that for the equilibrium between acridine and the acridinium cation [pKa = 5.6 (6)] while that for the equi-

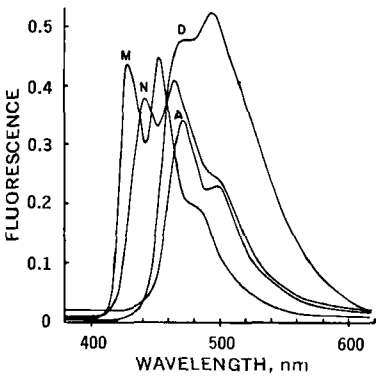


Figure 2—*Fluorescence spectra of the dication (D), monocation (M), neutral species (N), and anion (A) derived from 9-aminoacridine at room temperature.*

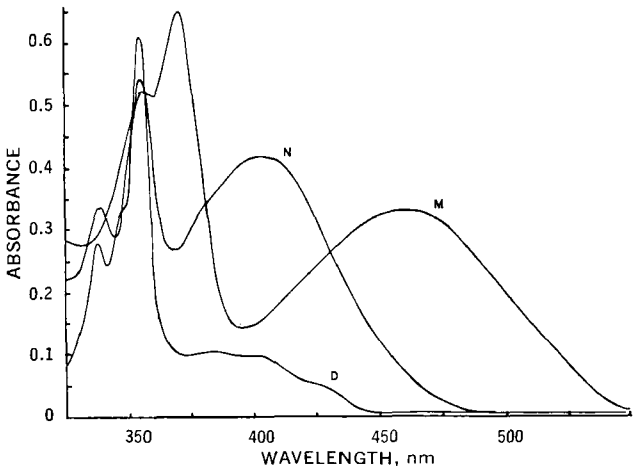


Figure 3—*Electronic absorption spectra of the dication (D), monocation (M), and neutral species (N) derived from 2-aminoacridine at room temperature.*

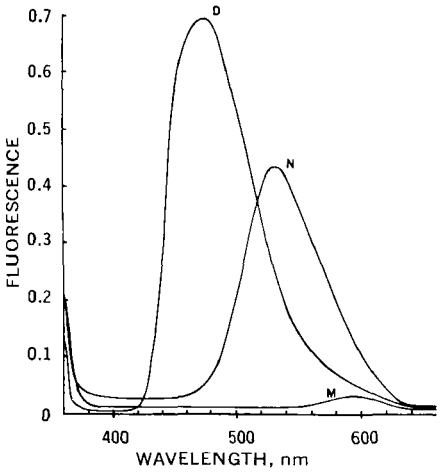


Figure 4—*Fluorescence spectra of the dication (D), monocation (M), and neutral species (N) derived from 2-aminoacridine at room temperature.*

librium between the singly and doubly charged cations is some three units more acidic than that for, say, 2-anthrylamine (12), a result of the protonated heterocyclic nitrogen atom in the acridine derivative.

The excited-state dissociation constant for the equilibrium between the singly and doubly charged cations derived from 2-aminoacridine is considerably more acidic than the corresponding ground-state dissociation constant. This, along with the previously described fluorescence behavior of 2-aminoacridine, indicates that this compound behaves as a typical *N*-heterocyclic amine in the lowest excited singlet state. The excited-state dissociation constant for the equilibrium between the singly charged cation and the neutral molecule could not be obtained by fluorometric titrimetry because the variations of intensities of the monocation and neutral molecule fluorescences with pH followed ground-state titration behavior. Apparently, the rates of fluorescence of the excited monocation and neutral molecule are too great for proton exchange with water to occur during the lifetimes of the lowest excited singlet state. However, in the pH 12–15 region, the fluorescence of the neutral molecule was quenched with increasing pH, although no change in the absorption spectrum of the neutral molecule was observed from pH 3 to 17.5. This behavior is attributed to ionization of the neutral amine in the lowest excited singlet state to form the excited iminate anion (Scheme I).

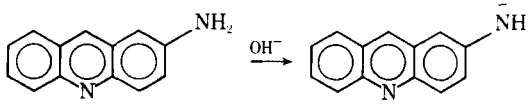
The failure of the absorption spectrum to change with increasing pH reflects that the ground-state iminate anion is too strong a base to be generated in aqueous solutions. This behavior, as well as the shift of the absorption and fluorescence spectra to longer wavelengths with increasing solvent polarity, is indicative of a much greater dipole moment of 2-aminoacridine in the lowest excited singlet state, the latter having a more charge-deficient amino group than in the ground state. This state of affairs is normal for arylamines.

The limited spectroscopic and pKa data available for the 1-, 3-, and 4-aminoacridines (13) suggest that these isomers also are "well-behaved" *N*-heterocyclic arylamines.

Table II—Ground (pKa) and Lowest Excited Singlet State (pKa*) Dissociation Constants for the Various Prototropic Equilibria of 2-Aminoacridine and 9-Aminoacridine[a]

	$D \rightleftarrows M + H^+$	$M \rightleftarrows N + H^+$	$N \rightleftarrows A + H^+$
2-Aminoacridine:			
pKa	1.1	5.9	—
pKa*	−5.8	—	12.4
9-Aminoacridine:			
pKa	−8.5	10.0	15.9
pKa*	−6.8	—	13.2

[a] The pKa values were determined by absorptiometric pH titrimetry. The pKa* values were determined by fluorometric pH titrimetry.



Scheme I

The absorption and fluorescence spectra of 9-aminoacridine and the acidity dependences of these spectra are quite different from those of 2-aminoacridine and are, in fact, similar to those of 4-aminoquinoline (7), the linear benzolog of 9-aminoacridine.

The absorption and fluorescence maxima of the monocation derived from 9-aminoacridine lie at wavelengths shorter than those of the acridinium cation as well as those of the monocation of 2-aminoacridine. This behavior is anomalous with regard to the expected spectral behavior of an amine-substituted aromatic ring(s) and suggests that the 9-aminoacridine is not an arylamine but rather the vinylog of a protonated amidine (protonated 9-iminoacridan). Similar spectroscopic behavior has been observed for protonated 9-aminoanthracene (12) relative to anthracene and for neutral acridone (8) (the oxygen analog of singly protonated 9-aminoacridine) relative to the acridinium cation and has been suggested to imply that protonated 9-aminoanthracene is a protonated imine rather than an arylammonium ion. The lactam structure of acridone is well known (6). The protonated heterocyclic nitrogen atom in 9-aminoacridine, which is more electronegative than the 10-carbon atom in 9-aminoanthracene, might be expected to encourage the "iminization" of the acridine derivative much as it does the lactonization of the 9-hydroxyacridine zwitterion to acridone.

The positive charge of the monocation thus appears to reside predominately at the exocyclic amino group. This conclusion is supported by the shifts of the absorption and fluorescence maxima of the monocation of 9-aminoacridine to shorter wavelengths with increasing solvent polarity. In well-behaved protonated heterocyclic amines, the spectra shift to longer wavelengths with increasing solvent polarity because the electronic dipole moment, which is directed from the amino group toward the highly electronegative, protonated, heterocyclic nitrogen atom, is increased in the lowest excited singlet state; the more polar solvent stabilizes the excited-state dipole relative to the ground-state dipole (10). The solvent shifts observed in the 9-aminoacridine monocation indicate that the ground state is relatively more stabilized than the excited state by increasing solvent polarity because the excited species has a lower dipole moment than the ground-state species. This is brought about by the flow of electronic charge away from the heterocyclic nitrogen atom and toward the exocyclic nitrogen atom in the excitation process and indicates that the exocyclic nitrogen atom is more electron withdrawing than the protonated heterocyclic nitrogen atom in singly protonated 9-aminoacridine.

Further evidence of the electron-withdrawing nature of the

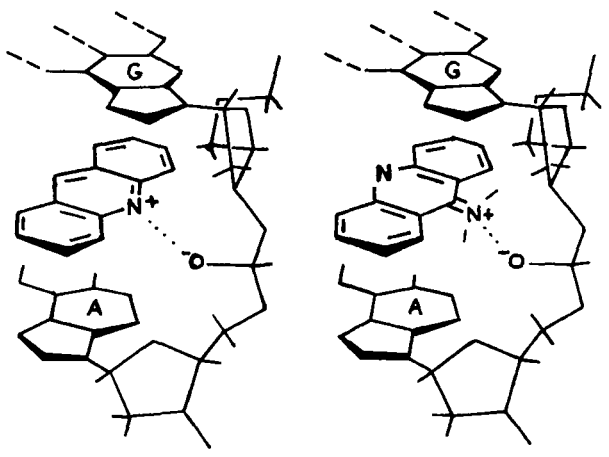


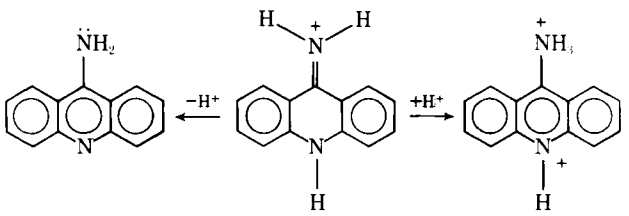
Figure 5—*Schematic illustration of:* (a) *the modified intercalation model with an unsubstituted acridinium cation intercalated between adjacent bases on a polynucleotide chain, and* (b) *the 9-aminoacridine monocation as the cyclic amidine in the same intercalation position.*

Table III—Electronic Absorption (λ_a) and Fluorescence (λ_f) Maxima of the Singly Charged Cations (M) and Neutral Molecules (N) Derived from 2-Aminoacridine, 9-Aminoacridine, and 10-Methyl-9-aminoacridinium Iodide in Solvents of Differing Polarity and Hydrogen-Bonding Capability

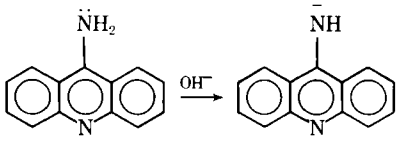
	Water		Ethanol		Dioxane		Chloroform		*n*-Heptane	
	λ_a, nm	λ_f, nm	λ_a, nm	λ_f, nm	λ_a, nm	λ_f, nm	λ_a, nm	λ_f, nm	λ_a, nm	λ_f, nm
2-Aminoacridine:										
M (maximum)	460	592	470	550	475	558	460	540	480	500
N (maximum)	405	531	422	521	415	497	405	481	396	439
9-Aminoacridine:										
M (O–O band)	423	430	425	430	425	435	425	434	—	—
(maximum)	402	455	405	456	405	459	405	458	405	460
N (O–O band)	426	445	425	430	425	435	424	434	418	440
(maximum)	406	470	403	455	404	459	403	458	400	468

amino group and the electron-donating nature of the heterocyclic nitrogen atom of the 9-aminoacridine monocation is seen in the changes of the absorption and fluorescence spectra of the monocation accompanying dissociation from the heterocyclic nitrogen atom and protonation of the exocyclic nitrogen atom (Scheme II). Dissociation of the monocation, which entails loss of a proton from the heterocyclic nitrogen atom, shifts the electronic spectra to longer wavelengths, a phenomenon that is anomalous for dissociation from nitrogen atoms in six-membered heterocyclic rings (9–11). This type of spectral shift indicates that the excited state is stabilized by a loss of positive charge from the heterocyclic nitrogen atom and is commonly observed in protolytic dissociation from electron donor groups such as —OH and —NH_2 (14). This suggests that the heterocyclic nitrogen atom of the 9-aminoacridine monocation is amine-like. Moreover, the pKa for the dissociation of the monocation is about four units higher than those for the dissociation of other aminoacridines (6), an observation that is difficult to reconcile with simple substituent perturbations. Protonation of the monocation to yield the dication results in spectral shifts to longer wavelengths, an observation that is impossible to reconcile with the withdrawal of two electrons from the aromatic system by protonation of the amino group. Furthermore, ground-state protonation of the monocation occurs in media about nine orders of magnitude more acidic than for any other aminoacridine monocation while excited-state protonation of the 9-aminoacridine monocation occurs in solutions less acidic than those in which ground-state protonation occurs. These observations are reminiscent of the prototropic and spectral behavior of 2- and 4-aminoquinolines (7) and strongly support the contention that the amino group is the site of positive charge in the 9-aminoacridine monocation.

The exact nature of the neutral species derived from 9-aminoacridine is somewhat more difficult to establish than that of the monocation. Nonaqueous IR studies (15, 16) have yielded conflicting conclusions as to whether the neutral amine has imine-like character. However, at least one study in aqueous media suggests that an imino tautomer may comprise about 10% of the total neutral 9-aminoacridine population (17). In very concentrated alkali solutions, the absorption and fluorescence spectra of the neutral species shift to longer wavelengths due to, in our opinion, the dissociation of the amino group (Scheme III).



Scheme II



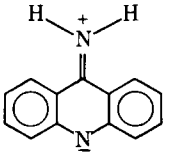
Scheme III

The shifts of the spectra to longer wavelengths upon dissociation and the fact that the fluorescence spectra shift at lower pH than the absorption spectra (*i.e.*, pKa* < pKa) are typical of arylamines. However, in ordinary arylamines the ground-state dissociation normally occurs at a pH too basic to be accessible and the shifts are normally much greater than observed here. To our knowledge, this is the first time that the dissociation of a neutral amine in the ground state has been observed in aqueous media.

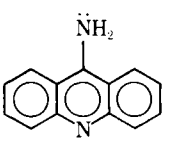
The spectra of the neutral 9-aminoacridine are amine-like in that they show a trend toward longer wavelengths with increasing solvent polarity. However, the shifts are much smaller than in other heterocyclic amines. This and the ease of ionization of the neutral amine in the ground electronic state suggest that even in the neutral molecule the amino group is somewhat charge deficient and that its actual electronic structure is intermediate between the zwitterionic structure (III) and the true amino form (IV).

The intercalation model proposed by Lerman (1, 2) depicted the ligand aminoacridine molecule, proflavin, as binding to double-helical DNA by insertion between, and centered over, adjacent base pairs. The amino groups are in the 3,6-position, each being directed toward a negatively charged phosphate ester linkage in the sugar backbone of each polynucleotide chain (3), and the long axis of the aminoacridine cation is nearly parallel to the hydrogen bonds of the nearest base pairs. The model did not explain how acridinium ions and singly protonated molecules with the 9-aminoacridine nucleus that have a bulky group attached at the 9-amino function, such as quinacrine (18), were able to intercalate with double-helical DNA. This consideration and others led to the proposal of a modified intercalation model (Fig. 5*a*), which called for the insertion of the acridine or 9-aminoacridine cation between adjacent bases on one of the polynucleotide chains comprising the double helix (4). The positive charge on the cation is closely associated with the negatively charged polynucleotide phosphate group on the same chain, such that the long axis of the cation is nearly perpendicular to the hydrogen bonds of the nearest base pairs. The position of the 9-carbon atom on acridine to which an amino group may be attached is no longer in the center of the helix, as dictated by the Lerman model, but is positioned to allow the amino group and bulky groups thereon access to the exterior of the helix. These two models apparently illustrate the two extremes that ligand molecules can assume upon intercalation into double-helical DNA.

Localization of the positive charge of protonation on exocyclic nitrogen should cause the 9-aminoacridine monocation, described by the modified intercalation model, to be "flipped over" so that positively charged exocyclic nitrogen is closely associated with the negatively charged polynucleotide phosphate group (Fig. 5*b*). When the monocation is in this position, bulky substituents on the exocyclic nitrogen, as found in quinacrine, would not seriously hamper intercalation by steric hindrance because they would have access to the exterior of the helix. However, some rotation or repositioning of the monocation about the point of ionic interaction



III



IV

could be expected due to the steric effect of bulky substituents attached at different sites on the aromatic nucleus or on the exocyclic nitrogen. The concentration of positive charge in 9-aminoacridinium cations at the exocyclic nitrogen atom also appears to be in agreement with the observation that 2,7-di-*tert*-butyl-9-aminoacridinium monocation intercalates with DNA (5), while 2,7-di-*tert*-butylproflavin monocation does not (19). In the former compound the bulky *tert*-butyl groups are directed toward the exterior of the helix where steric hindrance is minimal. In the proflavin derivative, however, the *tert*-butyl groups would be directed toward the interior of the helix if intercalation occurred, resulting in a strongly destabilizing steric interaction.

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Correlation and Prediction of Mass Transport across Membranes II: Influence of Vehicle Polarity on Flux from Solutions and Suspensions

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Abstract □ The effects of systematic alteration of vehicle composition on the release rate of drugs from their vehicle were studied. Equations were developed which quantitatively predict the rate of transport of a drug across a membrane separating two identical binary aqueous solvents. Separate equations were derived for solutions and for suspensions, which account for both the resistance of the membrane and the resistance of the solvent to drug diffusion.

Keyphrases □ Membrane diffusion—effect of vehicle polarity on flux from solutions and suspensions, systematic alteration of vehicle composition, equations □ Vehicle polarity—effect of systematic alteration of vehicle composition on release rates of drugs, equations □ Drug release rates—effects of systematic alteration of vehicle composition, solutions and suspensions, equations

One significant factor governing drug activity is the release rate of the drug from its vehicle. For nonsolid dosage forms (fluids, ointments, *etc.*), the ability of the vehicle to retain the drug can be conveniently altered by the addition of a second fluid, which may be a solvent or a nonsolvent for the drug. Of the many recent papers dealing with drug release from fluid vehicles, few have attempted to study, generalize, and quantitate the effects of incremental changes in vehicle composition systematically. The work of Poulsen (1) is a notable exception. In this regard, the authors have attempted to show the effects of vehicle composition upon solubility, the membrane vehicle partition coefficient, and, ultimately, the drug release rate.

THEORETICAL

Dependence of Flux upon Solubility and Partition Coefficient—The resistance, R_m, of a membrane to transport of a substance is proportional to the membrane's thickness, h_m, and inversely proportional to the diffusivity, D_m, of the substance in the membrane:

$$R_m = \frac{h_m}{D_m} \qquad \text{(Eq. 1)}$$

If the membrane separates two similar solvent phases, the regions of unstirred solvent (diffusion layers) adjacent to the membrane also offer resistance to the transport of a solute from the donor to the receptor phase. This solvent resistance is given by:

$$R_s = \frac{h_s}{D_s} \qquad \text{(Eq. 2)}$$