Fluorescence Emission Properties of the Cation of 4-Aminopyrazole(3,4-d)pyrimidine, an Adenine Analogue: Evidence for Phototautomerism

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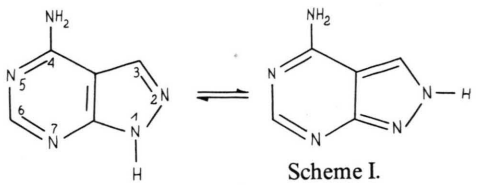
Fluorescence, Phototautomerism, Pyrazolo(3,4-d)pyrimidines, Adenine Analogues, Protonation Sites

A study has been made of the emission spectra at room temperature, in aqueous and alcoholic media, of 4-aminopyrazolo(3,4-d)pyrimidine (APP) and some of its methylated derivatives. The cationic forms $APPH^+$, N_2-methyl-$APPH^+$ and N_7-methyl-$APPH^+$ exhibit intense fluorescence under these conditions, the first two exhibiting excitation spectra which differ from the absorption spectra, pointing to the existence of a tautomeric equilibrium in the ground state. From the shape of the excitation spectra, and comparisons with methylated analogues in fixed tautomeric forms, it follows that the emission of $APPH^+$ originates exclusively from the species N(2)-H,N(7)-H^+, the other forms being non-fluorescent. The proportion of the emitting species, calculated from the excitation wavelength dependence of the quantum yield, is in good agreement with data for the ground state.

The emission spectrum of $APPH^+$ in aqueous medium consists of two bands with λ_{max} 360 nm and 430 nm, which exhibit identical excitation spectra, but are quenched to different extents by H_3O^+. The 430 nm emission band is absent in alcoholic media. A similar behaviour is exhibited by N_7-methyl-$APPH^+$, whereas the neutral form of this analogue exhibits only the 430 nm band. These results indicate that the long wavelength emission band of $APPH^+$ originates from the rare tautomeric species N(7)-H formed in the excited state by photodissociation of the N(2)-H proton from the species N(2)-H,N(7)-H^+. This is further confirmed by results obtained with the aid of the basicity method, as well as by salt efects in non-aqueous media. Consideration is given to the possibility of such processes occurring in other analogues of nucleic acid derivatives.

Considerable attention has been devoted to studies on the excited states of nucleic acids and their component purine and pyrimidine derivatives (for review, see Daniels [1]), as well as some of their more fluorescent analogues, in part with a view to their use as fluorescent probes in biological systems, *e. g.* 3-β-D-ribofuranosyl-7-aminopyrazolo(4,3-d)pyrimidine, or formycin A [2, 3], N_1,N^6-ethenoadenosine [4, 5], etc.

During the course of an investigation on the emission properties of analogues of 7-aminopyrazolo-(4,3-d)pyrimidine, the aglycone of formycin A (to be reported elsewhere), we found that the cationic forms of some of these exhibited anomalous behaviour in that their emission spectra included long-wavelength, broad, bands which did not correspond to the emission of model compounds. Subsequently we found that a similar behaviour, much more pronounced, is exhibited by the isomeric cation of 4-aminopyrazolo(3,4-d)pyrimidine (APP, see Scheme 1), a known inhibitor of purine biosynthesis. Both the foregoing pyrazolopyrimidines are isomers of adenine, a component of both RNA and DNA.



Scheme I.

We now proceed to demonstrate that the anomalous emission of the APP cation may be interpreted in terms of phototautomerization, which has been previously postulated for purines and pyrimidines from theoretical considerations, although with discordant results [6, 7]. One system, structurally simi-

Abbreviations employed: APP, 4-aminopyrazolo(3,4-d)pyrimidine; N_1-*m*-APP, 1-methyl-APP; N^4,N_7-m_2-APP, N^4,N_7-dimethyl-APP; and similar connotations for other methylated derivatives of APP; UV, ultraviolet, NMR, nuclear magnetic resonance; S_1 state, first excited singlet state; λ_{exc}, wavelength of excitation; φ, quantum yield; ε, molar extinction coefficient.

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lar to purines, which has been shown experimentally to exhibit phototautomerism, 7-azaindole, has been the subject of a number of investigations [8, 9, 10], although the validity of this model is not fully established. In the present instance we show that this problem is closely linked with the acid-base properties of the excited states of this class of compounds and the problem of deactivation of excited states of nucleic acid components.

Materials and Methods

APP was prepared according to a known method [11]. The N_5- and N_7-methyl derivatives were prepared by treatment of APP with methyl iodide in an excess of dimethylformamide [12], and the isolated products crystallized from water, m.p. 260 °C and 272 °C, respectively. Dodin *et al.* [12] reported 240 °C and 252 °C. Their identity was established by mass spectrometry ($m/e=149$), the spectrally determined pK_a values for protonation and/or dissociation, which were identical to those reported by Dodin *et al.* [12], and the rapid Dimroth rearrangement observed for the product identified as N_5-*m*-APP, but not for N_7-*m*-APP.

The N_1- and N_2-methyl derivatives were obtained by treatment of APP with diazomethane, as elsewhere described for the analogous 7-aminopyrazolo-(4,3-d)pyrimidine [13]. The melting points, pK_a values, and UV absorption spectra for both of these were in agreement with those reported for the same products obtained by a different route [14, 15]. An additional product obtained from this reaction, N_7-*m*-APP, was identical to that described in the previous paragraph.

The N^4-methylamino derivative of APP was prepared by the Dimroth rearrangement of N_5-*m*-APP, the resulting product being identical with that reported *via* another procedure [11]. Treatment of this product with methyl iodide (on a small scale) yielded a new compound which, on the basis of its UV spectrum, pK value, and intense blue emission in alkaline medium, was identified as N^4,N_7-m_2-APP.

All measurements utilized water doubly-distilled from quartz, redistilled analytical grade methanol and isopropanol from Merck (Darmstadt, Bundesrepublik Deutschland), and anhydrous formic and acetic acids from Fluka (Zürich). Salts were recrystallized, where necessary, to remove fluorescent impurities.

Absorption spectra were obtained with Zeiss (Jena, GDR) Specord UV-VIS and VSU-2P instruments. Emission and excitation spectra were recorded on an Aminco-Bowman SPF fitted with a Hanovia 901C xenon lamp and an RCA 1P28 photomultiplier. Excitation spectra were corrected by the method of Parker [16]. Emission spectra were corrected only for measurements of quantum yields and calculations of radiative constants. Yields were determined relative to quinine sulphate, $\varphi=0.55$ [17] and tryptophane, $\varphi=0.12$ [18]. Samples were not deaerated.

All of the compounds examined were checked for radiation sensitivity under the conditions employed in this investigation. Using the 1-methyluracil photohydration reaction as a chemical actinometer, is was established that the compounds were radiation resistant, with $\varphi < 10^{-4}$.

Results and Discussion

Fluorescent tautomers of APP and APPH$^+$

Elucidation of the emission properties of APP, as well as its cationic form ($APPH^+$), requires a knowledge of the tautomeric form(s) of the ground state and the emission properties of the individual form(s). The present study profited from the results of a recent investigation by Dodin *et al.* [12] on the tautomerism of these compounds by means of ^{13}C NMR spectroscopy and relaxation methods. In several instances, our findings could be used to check the validity of the foregoing.

The neutral form of APP, in contrast to its cation, exhibits very weak fluorescence at room temperature. A qualitative comparison of the UV spectrum of APP with those of its N-methyl derivatives showed that the principal tautomeric form is N(1)-H. This form is non-fluorescent, since N_1-*m*-APP exhibits no detectable emission at 300 °K. By contrast, N_2-*m*-APP emits weakly ($\varphi \sim 10^{-3}$); this is the fixed tautomeric form corresponding to the species N(2)-H of APP, the population of which was estimated by Dodin *et al.* [12] as about 10%. This form is proposed as the source of the weak emission of APP. The tautomeric form N(7)-H is apparently not present; if it were, it should be readily detectable because of its intense blue emission (see below).

With the aid of ^{13}C NMR spectroscopy, it has been established that the ground state of the APP cation consists of an equilibrium mixture of comparable proportions of at least three tautomeric forms, *viz.* N(1)-H, N(5)-H$^+$; N(2)-H, N(5)-H$^+$ and N(2)-H, N(7)-H$^+$. In order to establish which of these, or other, form(s) are responsible for the room-temperature emission of the APP cation, we have examined the emission properties of protonated N-methyl derivatives of APP.

The results of Dodin *et al.* [12] suggest that N_1-*m*-APP protonates almost exclusively on the ring N(5). The fluorescence of the cation of this compound in aqueous medium, with a maximum at 365 nm, is very weak (Table I). In methanolic medium, where the emission is somewhat more intense, the excitation spectrum differs appreciably from the absorption spectrum, reflected in the marked dependence of the fluorescence quantum yield on λ_{exc}, the yield for excitation at 270 nm being 20-fold lower than at 290 nm. One interpretation of this is that the emission originates from a minor tautomeric form, protonated on N(7), the proportion of which was evaluated by Dodin *et al.* [12] as about 2%. This is supported by the fact that, in contrast to N_7-*m*-APPH$^+$, the cation of N_5-*m*-APP exhibits no detectable emission at room temperature, indicating that the form N(1)-H, N(5)-H$^+$ is non-fluorescent under these conditions.

In contrast to the cation of N_1-*m*-APP, N_2-*m*-APPH$^+$ exhibits intense emission, with a maximum at about 360 nm in aqueous medium, and about 355 nm in alcoholic media. The emission spectrum is independent of λ_{exc}, but the quantum yield decreases significantly with a decrease in λ_{exc}, an effect particularly pronounced in aqueous medium

Table I. Location of fluorescence emission maxima, and quantum yields at various excitation wavelengths at room temperature.

Compound	Solvent	Emission λ_{exc} [nm]	λ_{max} [nm]	φ [a]	φ/φ_{max} [b]
N_1-*m*-APPH$^+$	0.01 N H_2SO_4/H_2O	280	365	0.002	
	0.01 N H_2SO_4/CH_3OH	290	360	0.015	1.0
		280	360	–	0.11
		270	360	–	0.05
N_2-*m*-APPH$^+$	0.01 N H_2SO_4/H_2O	300	360	0.072	1.0
		290	360	–	0.67
		280	360	–	0.44
		270	360	–	0.31
	0.01 N D_2SO_4/D_2O	300	355	0.075	
	0.01 N H_2SO_4/isopropanol	300	355	0.070	1.0
		270		–	0.87
N_7-*m*-APP	0.002 N KOH/H_2O	260–320	430	0.16	
	0.002 N KOH/CH_3OH	260–320	425	0.15	
N_7-*m*-APPH$^+$	0.001 N H_2SO_4/H_2O	290	365 [c]	0.025	1.0
			430 [c]	0.095	1.0
		260	365, 430 [c]	–	0.9 [d]
	0.001 N D_2SO_4/D_2O	290	365 [c]	0.054	
			430 [c]	0.082	
	0.001 N H_2SO_4/CH_3OH	260–300	360	0.19	
APPH$^+$	0.001 N H_2SO_4/H_2O	300	360 [c]	0.011	1.0
			430 [c]	0.041	1.0
		280	360, 430 [c]	–	0.43 [d]
		270	360, 430 [c]	–	0.26 [d]
		260	360, 430 [c]	–	0.19 [d]
	0.001 N D_2SO_4/D_2O	300	360 [c]	0.030	
			430 [c]	0.06	
	0.001 N H_2SO_4/isopropanol	290–300	355	0.077	1.0
		270	355	–	0.33
		260	355	–	0.24

[a] Error about 10%, except for N_1-*m*-APPH$^+$ (about 30%).
[b] Error about 5%.
[c] See text for method of resolution of these bands.
[d] This value is identical for both bands.

(Table I). This situation is reminiscent, and typical, for the coexistence of two (or more) forms, only one of which is fluorescent.

Excitation wavelength-dependence of fluorescence quantum yields, and discrepancies between absorption and excitation spectra, have been frequently reported for purine and pyrimidine derivatives [1, 19] and their cations [20], but their relevance to tautomerism has been questioned in a number of instances [19, 21]. In the present case there is little doubt that the behaviour of the N_2-*m*-APP cation is due to tautomerism, since the excitation spectrum closely resembles the absorption spectrum of the model analogue N_7-*m*-APPH$^+$ (Fig. 1) which, in turn, exhibits similar absorption and fluorescence excitation spectra.

Furthermore, the relative proportion of the emitting tautomer may be estimated quantitatively from the wavelength-dependence of the quantum yield, and compared with the ground-state data. Assuming that the excitation spectrum corresponds to the absorption spectrum of the fluorescent form N(7)-H$^+$, and that the actual quantum yield for this form is equal to the maximal value of φ at the extreme long-wavelength value of λ_{exc} (300 nm), the proportion of this form is:

$$\frac{[N(7)H^+]}{[N(7)H^+]+[N(5)H^+]} = \frac{\varphi(\lambda_{exc})}{\varphi_{max}} \cdot \frac{\varepsilon(\lambda_{exc})}{\varepsilon_{N(7)H^+}(\lambda_{exc})}.$$

If we take the value of ε_{max} for the form N(7)-H$^+$ as equal to that for N_7-*m*-APPH$^+$, we find that 37%±5% of the N_2-*m*-APP cation is in the fluorescent form (see Table I). This is in reasonably good agreement with the value of about 50% estimated by Dodin *et al.* [12], bearing in mind that the results obtained by ^{13}C NMR spectroscopy are, at best, only semi-quantitative (see ref. [22]), and confirming our interpretation of the emission properties of N_2-*m*-APPH$^+$.

It is of interest that, in acidified alcoholic medium, there is a marked enhancement in fluorescence quantum yield of N_2-*m*-APPH$^+$, particularly for $\lambda_{exc} < 280$ nm, with no essential change in location of the emission spectrum or shape of the excitation spectra. There is, on the other hand, a marked change in the absorption spectrum (Fig. 1, insert) which, in anhydrous isopropanol, now resembles the fluorescence excitation spectrum. It follows that, under these conditions, the tautomeric equilibrium is shifted in favour of the fluorescent form, to the extent of about 90% (see Table I).

From the foregoing results it may be anticipated that the emission of APP cation at 300 K originates from only one tautomeric species, *viz.* the form N(2)-H,N(7)-H$^+$. In fact, despite the differences between their absorption spectra, the fluorescence excitation spectra of N_2-*m*-APPH$^+$, APPH$^+$ and N_7-*m*-APPH$^+$ are closely similar in both aqueous and

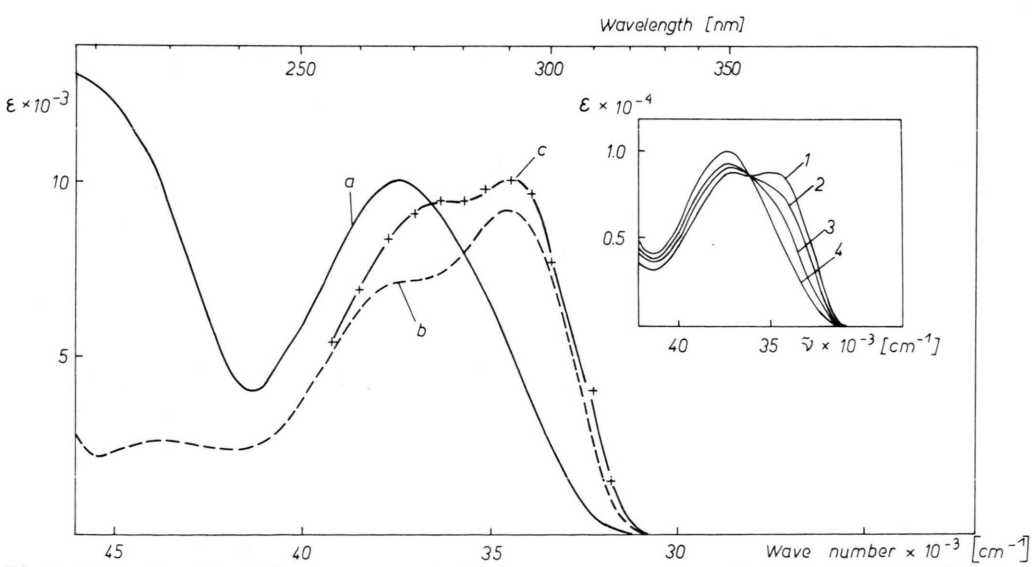


Fig. 1. Absorption (a) and fluorescence excitation (c) spectra of N_2-*m*-APPH$^+$ in aqueous 0.01 N H_2SO_4; and, for purposes of comparison, the absorption spectrum of N_7-*m*-APPH$^+$ (b) under the same conditions. *Insert:* Absorption spectrum of N_2-*m*-APPH$^+$ in the presence of 0.01 N H_2SO_4 in (1) anhydrous isopropanol, (2) isopropanol containing 5% water, (3) isopropanol containing 15% water, (4) in water.

non-aqueous media. Furthermore, the proportion of the fluorescent form of $APPH^+$, calculated from the excitation wavelength dependence of the fluorescence quantum yield (data in Table I), comes to about 20%. This is to be compared with the value of 20% for the N(2)-H,N(7)-H^+ tautomer of the APP cation in aqueous medium estimated from ^{13}C NMR spectroscopy by Dodin *et al.* [12].

The emission spectra of N_2-*m*-$APPH^+$, $APPH^+$ and N_7-*m*-$APPH^+$ are also similar in alcoholic media, except for the higher intensity of the latter (Table I). Surprisingly, however, they differ appreciably in aqueous medium (see below). From considerations cited above, and particularly in view of the identity of their excitation spectra in both aqueous and alcoholic media, it follows that the foregoing differences between the emission spectra in aqueous medium must be ascribed to differences in the excited state structures of these compounds. We shall revert to this question below.

It should be emphasized that, for the compounds considered above, the pK_a values for protonation, measured by emission and absorption spectroscopy, were identical. Hence the observed changes in fluorescence are determined by acid-base equilibria in the ground state. This does not, however, necessarily exclude some involvement of excited state prototropic processes. Identity of pK_a values from emission and absorption spectroscopy does not, as suggested by Wilson *et al.* [19], constitute an argument against the origin of emission from minor tautomeric species. The ground state is one corresponding to complete thermodynamic equilibrium, and the molar concentrations of all, including minor, tautomeric forms should exhibit the same pH-dependence, *i.e.* exhibit a common pK_a value, not necessarily the same as the microscopic pK values of the individual species. But this will not necessarily hold for the excited state (see below).

Emission of APP cation in aqueous medium

In the presence of 0.001 N H_2SO_4 the emission of $APPH^+$ in aqueous medium differs significantly from that in alcoholic media, and also from the emission of N_2-*m*-$APPH^+$ in water, in that it exhibits an additional band at 430 nm with an intensity much higher than the 360 nm band (visible only as an inflexion, Fig. 2). Further acidification leads to marked quenching of the 430 nm band, so that the 360 nm band becomes clearly defined (Fig. 2, curve b). In this pH range the absorption and excitation spectra are unchanged, so that the quenching of the 430 nm band must be considered as dynamic quenching. However, the relative quantum yields for the two bands are independent of λ_{exc} over this pH range; while the excitation spectra, identical for

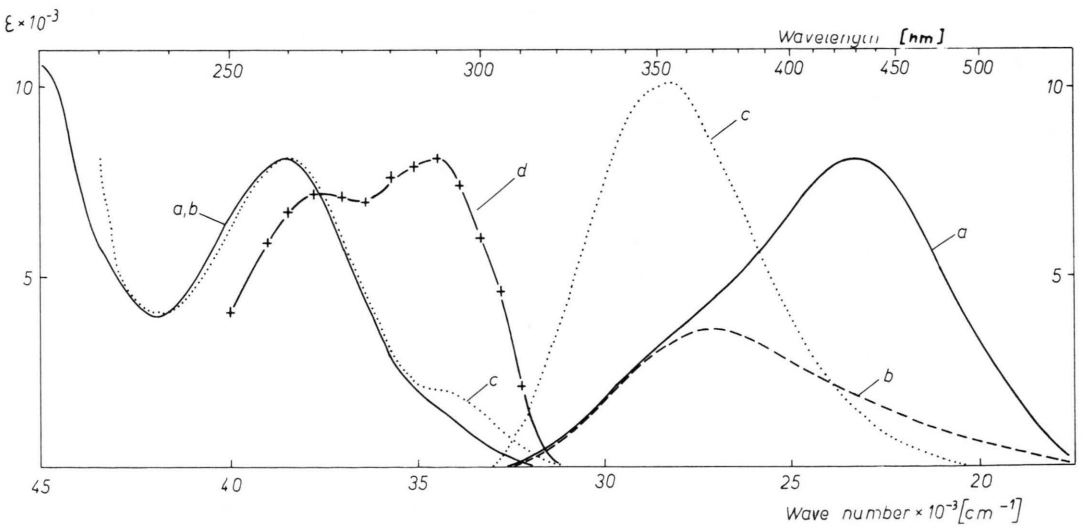


Fig. 2. Absorption, excitation and emission spectra of APP cation under various conditions: *Left-hand side:* (a, b) Absorption spectrum in aqueous 0.001 N and 0.1 N H_2SO_4, identical. (c) Absorption spectrum in 0.01 N H_2SO_4 in anhydrous isopropanol. (d) Fluorescence excitation spectrum in 0.001–0.1 N H_2SO_4 in isopropanol. *Right-hand side:* (a) Emission spectrum in aqueous 0.001 N H_2SO_4. (b) Emission spectrum in aqueous 0.1 N H_2SO_4. (c) Emission spectrum in 0.001–0.1 N H_2SO_4 in isopropanol.

both bands (see above), do not differ significantly from those in alcoholic media.

The foregoing effects were independent of the $APPH^+$ concentration over the range of $10^{-3}-10^{-6}$ M, thus excluding possible involvement of self-associates either in the ground or excited states.

The difference in behaviour of the two bands in the pH range 3 to 1 shows that they must originate from different emitting species. The identity of their excitation spectra indicates that both bands derive from a common absorbing species, in this case the tautomeric form N(2)-H,N(7)-H^+. It follows that the observed dual emission is due to a change in the structure of a fraction of the emitting molecules during the lifetime of the excited state.

We now proceed to an examination of N_7-*m*-APP, the properties of which, as shown below, provide an interpretation for the origin of the 430 nm emission band of $APPH^+$.

Emission properties of N_7-m-APP

Spectral titration showed this compound to be quite basic, pK_a for protonation 7.3. In aqueous medium, at pH > 9.3, the resulting neutral form exhibited intense emission centred at 430 nm, the location and band shape of which (Fig. 4, curve a) are virtually identical with the long-wavelength emission band of the APP cation (Fig. 2). The excitation spectrum is in excellent agreement with the absorption spectrum of the neutral species, so that absorption and emission derive from a single form. This is consistent with the exclusive amino structure postulated for this analogue by Dodin *et al.* [12].

However, the rather large Stokes' shift of the fluorescence band (~ 10000 cm^{-1}, Fig. 4) suggests some significant change in structure of the S_1 state relative to the ground state [23]. The absence of vibrational structure, even at 77 K, rendered difficult an analysis of these changes. But the identity of the emission spectrum (including intensity) in both aqueous and non-aqueous media point to the absence of proton migration in the excited state, so that the emission must originate from the neutral form of N_7-*m*-APP. The most reasonable interpretation of the large Stokes' shift is a change in geometry of the S_1 state; this is supported by the marked blue shift of the emission at low temperature, where the fluorescence maximum is centred at 360 nm (data not shown). Similar large Stokes' shifts have been reported for other compounds of this class [24a, 24b, 24c], but their origin in most cases has not been elucidated.

The protonated form of N_7-*m*-APP (in aqueous medium, pH 3) exhibited the same 430 nm emission band, but with a quantum yield only one-half that for the neutral form at pH > 9.3 (Fig. 4). In addition, the short-wavelength shoulder extended further to the violet, pointing to the existence of an additional, weaker, band centred at about 365 nm. With a further decrease in pH, the intensity of the 430 nm band diminished appreciably, while that of the short-wavelength band increased somewhat, showing a clearly defined maximum at 365 nm (Fig. 4,

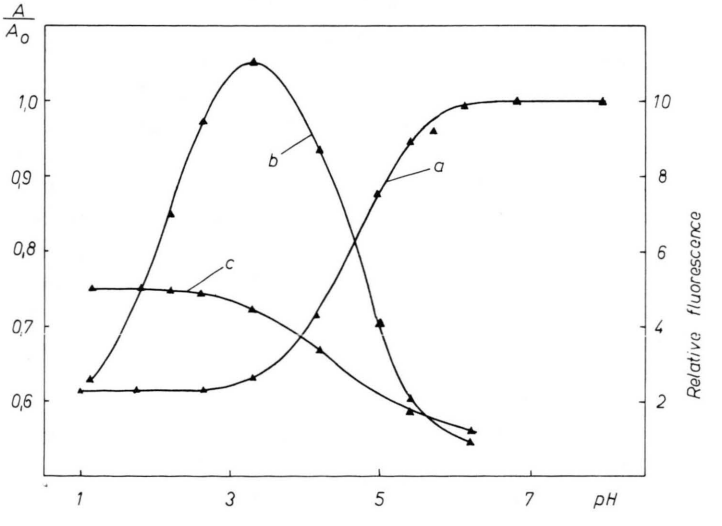


Fig. 3. Spectrophotometric and fluorometric titration of APP in aqueous medium: (a) Spectral titration, at $\lambda = 272$ nm. (b) Fluorimetric titration, recorded at 355 nm. (c) Fluorimetric titration, recorded at 430 nm. Excitation for fluorimetric titrations was at 260 nm (isosbestic point). The solutions were brought to pH values below 3.5 with H_2SO_4, and to other pH values with acetate and phosphate buffers at concentrations of ~ 10^{-3} M, which do not detectably quench fluorescence.

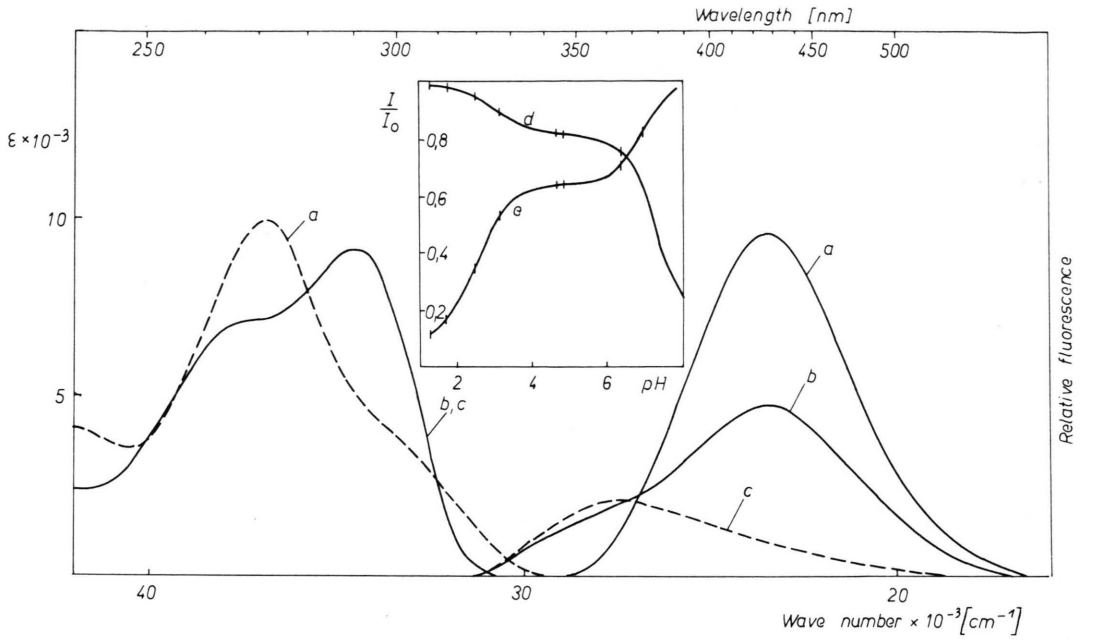


Fig. 4. Absorption and emission of N_7-*m*-APP in aqueous medium: *Left-hand side:* (a) Absorption spectrum in 0.002 N KOH. (b, c) Absorption spectra in 0.001 N and 0.1 N H_2SO_4, identical. *Right-hand side:* Emission spectra, independent of λ_{exc}, (a) in 0.002 N KOH, (b) in 0.001 N H_2SO_4, (c) in 0.1 N H_2SO_4. *Insert:* Fluorimetric titration curves, with excitation at 280 nm, recorded at (d) 355 nm, (e) 430 nm.

curve c). The absorption spectrum was unchanged over this pH range, as was also the excitation spectrum, which was identical for both bands, and similar to the absorption spectrum. It is clear that the emission spectrum of N_7-*m*-APP in acid medium (cationic form) is virtually identical with that for the APP cation under the same conditions (*cf.* Figs. 2 and 4), the only difference being in the quantum yields (Table I). The agreement between the absorption and fluorescent excitation spectra is consistent with the claim of Dodin *et al.* [12] that N_7-*m*-APP undergoes protonation almost exclusively on N(2).

The presence of the 430 nm emission band of the neutral form of N_7-*m*-APP in aqueous medium at pH values well below the pK_a for protonation (Fig. 4, insert) points to dissociation of the cation in the excited state. The basic criterion for this is the identity of the excitation spectrum for the long-wavelength band in acid medium with the absorption spectrum of the cation. On the other hand, the enhancement of the 365 nm emission band of the protonated form with decreasing pH was insufficient to satisfy the well-known relation $\varphi/\varphi_0 + \varphi'/\varphi'_0 = 1$ [25a, 25b]. This is readily explicable on the assumption that reprotonation, which should occur in more acid medium (pH ~ 2), leads to cationic form(s) other than those in the ground state, and non-fluorescent [26]. The dynamic quenching of the long-wavelength emission at about pH 2.5 is then due solely to the kinetics of (re)protonation of the fluorescent form. For this reason, it is not feasible to estimate a value for pK* by fluorimetric titration [27].

An approximate estimate of pK* may be obtained with the aid of the Foerster cycle [28, 29]. The absorption spectrum of the neutral form of N_7-*m*-APP exhibits a weak non-structured band visible as an inflection on the long-wavelength shoulder of the intense 270 nm band (Fig. 4). The λ_{max} of this weak band, following resolution of the spectrum by the procedure of Metzler [30], was 302–304 nm. Application of the Foerster equation,

$$pK_G - pK_{S_1} = (\tilde{\nu}_{00}^{neutr} - \tilde{\nu}_{00}^{cat})\, Nh/RT \ln 10$$

where the $\tilde{\nu}_{00}$ values were estimated from the average value of the half-height intensity points for the long-wavelength absorption and fluorescence bands

[29], gave $pK^* = 1.7$. This value indicates that dissociation of the excited cation of N_7-*m*-APP is energetically feasible.

In anhydrous methanolic medium, acidified to 0.001 N H_2SO_4, the N_7-*m*-APP cation exhibited only a 360 nm emission band, like N_2-*m*-$APPH^+$ and $APPH^+$, but in higher yield (Table I). The excitation spectrum coincided with the absorption spectrum. Hence photodissociation does not occur under these conditions. This is not unexpected, since alcohols are weaker proton acceptors than water, and are known to appreciably slow down such processes as phototautomerization [31] and photodissociation [32].

Effect of added salts

Addition to the methanolic medium of strong proton acceptors, such as ions of weak acids, led to restoration of photodissociation of N_7-*m*-$APPH^+$, as may be seen from Fig. 5 B. Formate ions (and, to an identical extent, acetate ions) led to quenching of cation emission and appearance of the emission of the neutral form. The Stern-Vollmer relation [25a] applies here, and the calculated quenching constant, 18 M^{-1}, divided by the life-time of N_7-*m*-$APPH^+$ in the S_1 state (~ 3 nsec, estimated from the radiative constant and the quantum yield, Table II) gave a value for the kinetic constant, $\sim 6\times10^9$ M^{-1} sec^{-1}, comparable to that for ion quenching of the emission of β-naphthol under comparable conditions (3.1×10^9 [33]), consistent with a diffusion-controlled process. It should be emphasized that the appearance of the long-wavelength emission in the foregoing case is not due to the presence of the neutral form in the ground state, since the excitation spectra for both observed bands (Fig. 5B) are identical and, furthermore, coincide with the absorption spectrum of the cation. The above effect consequently independently confirms the interpretation of the dual

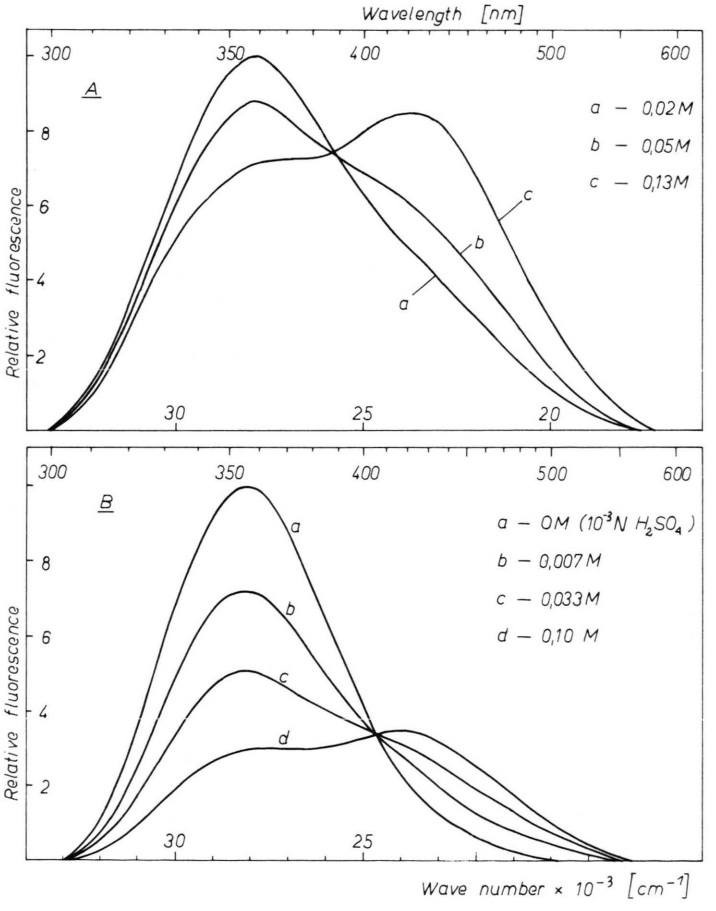


Fig. 5. Effect of various concentrations of formate buffer ($HCOOH$-$HCOONH_4$, 2 : 1) on the emission spectra of the cationic forms of (A) APP, and (B) N_7-*m*-APP in methanolic medium. Figures refer to molar concentrations of ammonium formate. The effect is independent of λ_{exc}. *Note:* the formic acid serves only to maintain constant pH of the medium, and by itself does not affect the fluorescence spectrum or intensity at concentrations up to 0.3 M (see Table II).

Table II. Calculated radiative constants and lifetimes of the S_1 states, and rate constants for ion catalyzed proton transfer.

Compound	Solvent	λ_{max} emission [nm]	k_{rad}^{-1} [nsec]	$\tau = k_{rad}^{-1} \cdot \varphi$ [nsec]	Quenching agent and quenching constant $K_{SV}[M^{-1}]$		$k_Q = K_{SV}/\tau$ $[M^{-1}\ sec^{-1}]$
N_7-*m*-APPH$^+$	CH_3OH	360	14 [a]	~ 3	CH_3COO^-	18	6×10^9
					$HCOO^-$	18.5	6×10^9
APPH$^+$	CH_3OH	360	[b]	~ 1	$HCOO^-$	5.6	5×10^9
N_2-*m*-APPH$^+$	CH_3OH	355	[b]	~ 1	$HCOO^-$	< 0.2	–
N_7-*m*-APP	H_2O	430	60 [a]	~ 9.5	CH_3COOH	0.5 [d]	5×10^7
				10 ± 1.5 [c]	HCOOH	3.9 [d]	4×10^8
					H_3O^+	~ 150	1.5×10^{10}
N_7-*m*-APP	CH_3OH	430		~ 9	HCOOH	< 0.5 [d]	–

[a] Calculated with the aid of the Strickler-Berg equation [46], following resolution of the absorption bands by the method of Metzler [30].
[b] This value was taken as equal to that for N_7-*m*-APPH$^+$.
[c] Measured by means of the impulse technique.
[d] Quenching of the long-wavelength emission at 430 nm was measured at pH ~ 4 in the presence of a constant concentration of $RCOO^-$ ions.

emission of the N_7-*m*-APP cation in *aqueous* medium.

As anticipated, addition of formate, or acetate, ions did not affect the emission of N_2-*m*-APPH$^+$. Photodissociation of this analogue is not possible, since there is no proton on the ring nitrogens of the pyrazole ring. From Fig. 5A, however, it will be seen that formate ions markedly affect the emission of the APP cation in methanolic medium. As in the case of N_7-*m*-APPH$^+$, there is quenching of the short-wavelength, 360 nm, band, and an increase in intensity of the long-wavelength band, with maintenance of an isoemissive point. The calculated quenching constant, 6 M^{-1}, is somewhat lower than for N_7-*m*-APPH$^+$, but, when divided by the calculated lifetime, leads to the same value for the kinetic quenching constant (Table II).

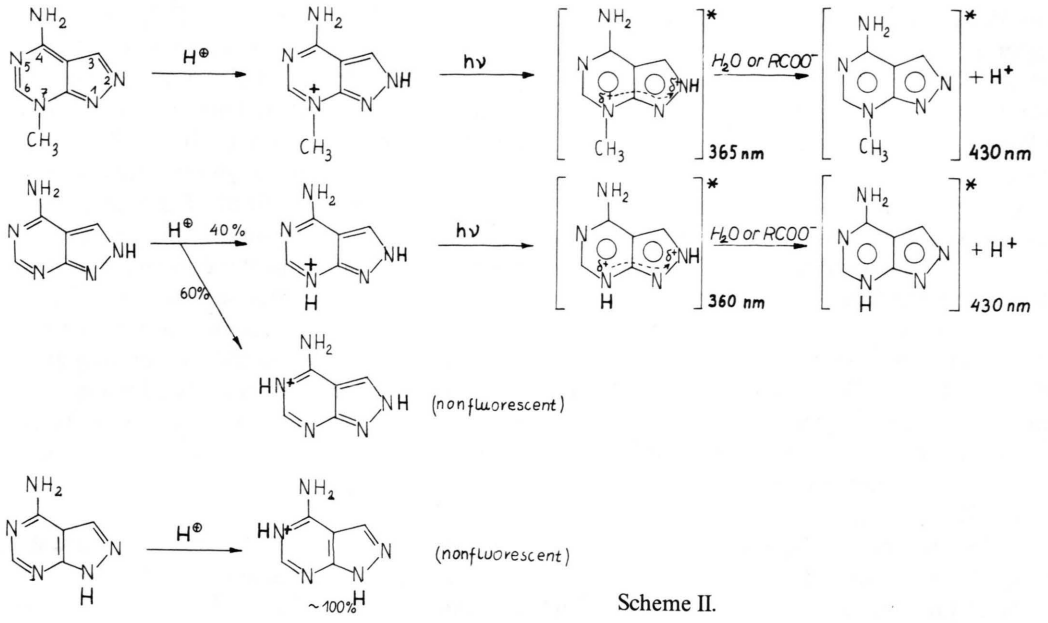
Interpretation of dual emission of APPH$^+$

The following data permit of the interpretation of the dual emission of the cations of APP and N_7-*m*-APP as shown in Scheme II.

From Scheme II, it is clear that the excited APP cation tends to release a proton from a pyrazole ring nitrogen to form the rare tautomer N(7)-H. This proposal is also consistent with thermodynamic data. The value of 1.7 for the pK* of N_7-*m*-APP (see above) may be considered as a microscopic pK* for the N(7)-H tautomer, when protonated on the ring N(2). Similarly, application of the Foerster cycle to N_2-*m*-APP gives a microscopic pK*, of approximately 6, for protonation of the N(2)-H tautomer on the ring N(7); this exceeds the pK_a value of 5.1 for the ground state, so that photodissociation of the proton on the ring N(7) is not possible. The basicity method may be applied in this case [34], and shows that the "rare" N(7)-H form in the S_1 state must be about 6 kcal/mol more stable than the N(2)-H tautomer.

While this scheme accounts for the origin of the dual emission, there is some anomaly in the behaviour of APPH$^+$ and N_7-*m*-APPH$^+$ in aqueous medium, as compared to that in methanolic medium. While ion quenching of the short-wavelength band is observed in both media, and the measured quenching constants are in agreement with diffusion-controlled kinetics, there is little or no increase in emission of the 430 nm band in aqueous medium. Measured quenching constants for the specific quenching of the 430 nm band by acids are not high enough (particularly for acetic acid, see Table II), to explain this phenomenon. Furthermore, an isotope effect which normally accompanies proton photodissociation [35], is observed only for the short wavelength band, with a magnitude comparable to that for typical examples of photodissociation (Table I). The emission of N_2-*m*-APPH$^+$, which is not quenched by ions, did not exhibit any isotope effect.

The foregoing anomaly does not invalidate the general proposed scheme. It is known that the course of excited state proton transfer may depend both on the solvent and the structure of the solva-



Scheme II.

tion shell, *e.g.* salicylic acid in ethanolic medium [36a, b]. The present example is additionally complicated by the proposed change in geometry of neutral N_7-*m*-APP molecule in the S_1 state (see above), which may also depend on the structure of the solvation sphere [37]. The ions may, in this instance, act not only as catalysts of proton transfer, but also by perturbing the structure of the solvent, and thus lead to the specific quenching of the emission. The phenomenon of diabatic proton transfer, *i.e.* with the loss of excitation energy, as pointed out by Ireland & Waytt [38], also cannot be excluded for this case.

It is of interest that two analogous derivatives, each with a methylated amino group, N^4-*m*-APPH$^+$ and N^4,N_7-m_2-APPH$^+$, do not exhibit the above anomaly, in that their behaviour is similar in both aqueous and methanolic media. In the absence of ions they exhibit weak fluorescence ($\varphi \sim 3\times10^{-3}$), with a maximum at about 360 nm. By contrast, addition of concentrated formate buffer (≥ 1 M) leads to the appearance, with both compounds, independently of the solvent, of a fairly intense band at 430 nm, identical with the emission band of the neutral form of N^4,N_7-m_2-APP. These facts once again support the validity of the mechanism presented in scheme II.

Concluding Remarks

As far as we are aware, this is the first reported instance of phototautomerism amongst analogues of purines and pyrimidines, and one of only a few instances of such phototautomerism in ring systems (apart from 7-azaindole and alloxazine [39a, 39b]). It is, however, probably not a rare exception, since a similar phenomenon has been encountered with the isomeric 7-aminopyrazolo(4,3-d)pyrimidines (to be published). Photodissociation, but not phototautomerism, has also been demonstrated for the cation of N_1,N^6-ethenoadenosine [4].

In contrast to most reported instances of phototautomerization, APP exhibits very low emission at room temperature ($\varphi < 10^{-3}$), corresponding to a lifetime for the S_1 state of the principal tautomeric form(s) of 10^{-11} to 10^{-12} sec. This accounts for the absence of phototautomerization in neutral medium. For the same reason, phototautomerization is excluded *via* cooperative transfer of two protons, such as observed for 7-azaindole in alcoholic media [10], and postulated for alloxazine [39a] and other systems [40]. A necessary condition for phototautomerization in APP is protonation of the molecule in the ground state (although it is conceivable that this may also proceed *via* the anion, since the emission maximum of the anionic form is red-

shifted in concentrated ammoniacal buffer). However, under appropriate conditions, excited state proton transfer may be a very rapid process, even without an energy barrier [41], so that such processes cannot be fully excluded, *e.g.* in strongly hydrogen bonded complexes.

The ability to observe phototautomerism in APP in acid medium is due to the favourable emission properties of the rare tautomeric form N(7)-H relative to other tautomeric forms, and likely due to reversal of the two lowest excited ($\pi-\pi^*$) singlet states. Whereas for the principal tautomeric forms, N(1)-H and N(2)-H, the $S_1 \leftarrow S_0$ transition is strong ($\varepsilon \sim 10000$) and exhibits weak vibrational structure at 300 K (quite distinct at 77 K), N_7-*m*-APP exhibits a weak ($\varepsilon \sim 3000$), broad band with no vibrational structure even at 77 K. These bands, as in the case of adenine [42], may be ascribed to L_a and L_b transitions, respectively. Attention should be drawn to the striking similarity of the emission properties of N_7-*m*-APP to ethenoadenosine [5], pointing to similarity of the emitting states in both compounds.

An analogous reversal of the sequence of the transitions L_a and L_b, accompanied by a marked decrease in energy of the latter, has been calculated for the rare imino tautomers of adenine [43] and observed in model compounds [44]. Application of the Foerster cycle in the case of 1,9-dimethyladenine, the cation of which corresponds to the principal tautomeric form of the cation of adenosine [45], points to the feasibility, energetically, of dissociation of an amino proton in the excited state. But it would be difficult to observe this experimentally, since the neutral form of the compound does not emit under normal conditions.

The foregoing results, and the experimental data for APP, suggest that processes of excited-state proton migration are *energetically* feasible for compounds of this class, and should be considered in studies on deactivation of their excited states. For purines and pyrimidines, however, the *kinetic* feasibility of these processes remains an open question.

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