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Fluorescence and Tautomerism of Protonated and Methylated Adenine Derivatives

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The fluorescence of adenine, adenosine, and adenosine-5′-phosphates has been studied in acid solutions between room temperature and 190°K. Discrepancies between absorption spectra and fluorescence excitation spectra showed that more than one tautomer was present in each solution. In order to characterize the tautomers, the analogous kationic methylated derivatives of adenine were investigated at pH 2. 1-Methyladenine, 3-methyladenine, and 3,7-dimethyladenine were devoid of fluorescence. 7-Methyladenine and 1-methyladenosine were weakly fluorescent, and again tautomerism was evident. It is concluded from comparisons of spectra and fluorescence properties that the well known protonation at N-1 in adenine does occur, but does not explain the fluorescence. The fluorescent tautomer of protonated adenine probably has 3 hydrogens in the extranuclear amino group and one hydrogen at N-7, while the other nitrogens retain their free electron pairs. In the case of adenosine no evidence was obtained for or against the existence of a tautomer with 3 protons in the amino group, since the fluorescence could be explained by protonation of N-1 in acid solutions.

Cooling did not greatly enhance the weak fluorescence of protonated and methylated derivatives of adenine. This is used as a basis for the hypothesis that these compounds emit their fluorescence from excited singlet states of $n \rightarrow \pi^*$ promotion type.

The present work shows that the protonated, kationic adenine ring exists in more than one tautomeric form in acid solutions. Several studies of crystalline substances by X-ray crystallography [1–3] and infrared spectrometry [4] have failed to reveal any evidence of tautomerism, and show that the positively charged adenine ring is invariably protonated at N-1, while the amino group does not comprise more than two hydrogen atoms. It is clear, however, that the absence of tautomerism in crystalline substances does not preclude tautomeric equilibria in the solutions from which the crystals grow. The equilibrium will, of course, be displaced towards the least soluble tautomer as crystallization proceeds.

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In the case of adenosine evidence against tautomerism in acid D_2O solution has been obtained by proton magnetic resonance which could "be understood in terms of a definite tautomeric structure of adenosine in which the hydrogen is attached to N-1...".[5] This somewhat vague statement probably means that the authors hesitate to exclude the possibility of alternative interpretations, *e.g.* the occurrence of minor amounts of other tautomers.

Quantum mechanical calculations [6] and the reactivities of adenine derivatives towards electrophilic reagents [7–9] indicate that N-1 and N-3 do not differ greatly in their affinity for protons and other electrophilic reagents. N-7 is less reactive, while the proton affinity of the amino group appears not to have been determined. Thus no decisive theoretical or experimental evidence against the occurrence of adenine derivatives in more than one tautomeric form has been reported, although there is good reason to believe that the tautomer which dominates quantitatively in acid solutions carries a proton bonded to N-1.

The occurrence of tautomerism in acid solutions having been demonstrated, the problem was to establish the structures of the fluorescent and nonfluorescent tautomers. For this purpose we have investigated adenine derivatives methylated in known positions. In order to be structurally equivalent to the protonated adenine derivatives, the methylated compounds were studied in their kationic form at pH 2.

In a previous publication [10] the fluorescence of adenine derivatives in acid solutions was supposed to be due to the well known protonation of N-1. It was assumed, furthermore, that this protonation changed the lowest excited singlet state from a nonfluorescent n,π^* state to a fluorescent π,π^* state, although it was shown that the faint fluorescence of pyrimidine from an excited singlet state of n,π^* type [10,11] caused this interpretation to be rather uncertain. These statements are re-evaluated in the present paper in view of the demonstrated tautomerism, and with reference to the experimental effects of cooling on the fluorescence yields of kationic adenine and its derivatives.

EXPERIMENTAL

Materials. Adenine was obtained from Hoffman-La Roche & Co. Ltd., Basle, Switzerland. Purine, pyrimidine, and the non-methylated derivatives of adenine were products of Nutritional Biochemicals Corporation, Cleveland, Ohio, U.S.A. The 1-, 3-, and 7-monomethyladenine as well as 3,7-dimethyladenine and 1-methyladenosine were kindly supplied by P. D. Lawley * and R. Denayer.** Methods of preparation are described in Refs. 7, 12, and 13. The fluorescence standard, quinine hydrogen sulfate, was purchased from The British Drug Houses, Ltd., Poole, England.

The purity and identity of adenine and its methylated derivatives were checked by paper chromatography on Whatman No. 3 paper in two solvent systems: butanol:aqueous ammonia, sp. gr. 0.88:water, 85:2:13, and methanol:conc. hydrochloric acid:water, 7:2:1 by volume. The R_F values were in agreement with the data in the literature,[8,9] and no fluorescent or UV-absorbing contaminations were found.

Procedure. The fluorescence spectrophotometer has been described previously.[14] Due to the low quantum yields of the luminescence phenomena observed, solutions with

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absorbances up to 0.4 cm^{-1} had to be used. The ensuing attenuation of the exciting light tended to depress the excitation spectra around the absorption maxima of the fluorescent solutions. An approximate correction for this filter effect was applied, based on the assumption that the fluorescence light seen by the photomultiplier was emitted from the center of the fluorescence cell.

RESULTS

Fluorescence excitation spectra. The fluorescence of protonated purine was regular in the sense that the fluorescence excitation spectrum coincided with the absorption spectrum within the range of possible experimental error (Fig. 1). Protonated adenine, adenosine, and adenosine phosphates all demonstrated, however, significant discrepancies between absorption spectra and fluorescence excitation spectra (Figs. 2—4). Such disagreements between the two types of spectra were found in aqueous solutions as well as in methanol: water mixtures (Fig. 4).

Among the methylated adenine derivatives 1- and 3-methyladenine and 3,7-dimethyladenine were nonfluorescent. 7-Methyladenine and 1-methyladenosine were weakly fluorescent. They exhibited differences between absorption spectra and fluorescence excitation spectra similar to those described above for the protonated adenine derivatives (Figs. 5 and 6).

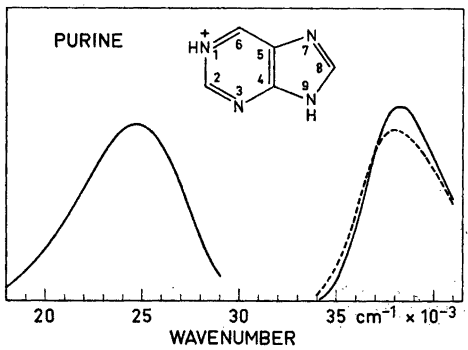

Fig. 1. Fluorescence of purine in aqueous solution at room temperature. $[H_2SO_4] = 10^{-2}$ N. The unit along the ordinate of the corrected emission spectrum (left) is: Number of quanta emitted per unit time per unit wavenumber interval. The solid curve in the right half of the figure is the absorption spectrum of the fluorescent solution (linear absorbance scale). The stippled curve is the fluorescence excitation spectrum corrected for instrumental artefacts and inner filter effect due to the non-negligible absorbance (absorbance = 0.125 at 38 000^{-1} cm and 1 cm light path).

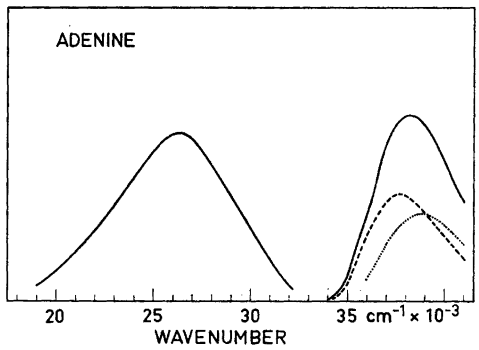

Fig. 2. Fluorescence of adenine in aqueous solution at room temperature. $[H_2SO_4] = 10^{-2}$ N. Absorbance at 38 000 $cm^{-1} = 0.32$, light path 1 cm. Same units and meaning of symbols as in Fig. 1. The dotted curve is the estimated absorption spectrum of the nonfluorescent tautomer, obtained as the difference between the total absorption spectrum of the solution and the fluorescence excitation spectrum of the fluorescent tautomer.

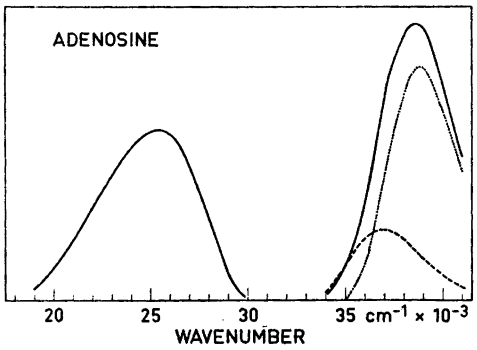

Fig. 3. Fluorescence of adenosine in methanol:water 10:1 (v/v) at room temperature. $[H_2SO_4] = 10^{-2}$ N. Absorbance at 38 500 $cm^{-1} = 0.35$, light path 1 cm. Same units and meaning of curve symbols as in Figs. 1 and 2.

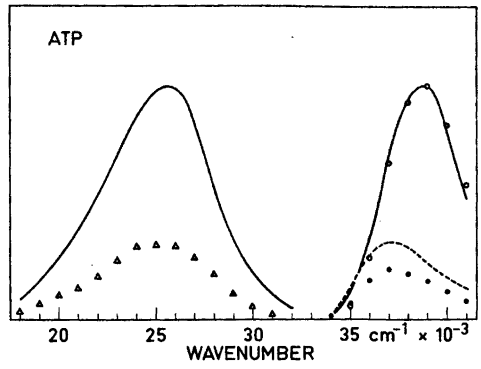

Fig. 4. Fluorescence of adenosine-5′-triphosphate (ATP) at room temperature in aqueous solution (points) and in methanol: water 10:1 (v/v) (curves). $[H_2SO_4] = 10^{-2}$ N. Absorbance at 39 000 $cm^{-1} = 0.20$, light path 1 cm. Same units and meaning of curve symbols as in Fig. 1. Filled circles represent the fluorescence excitation spectrum, open circles the absorption spectrum, and open triangles the emission spectrum of the fluorescent (aqueous) solution.

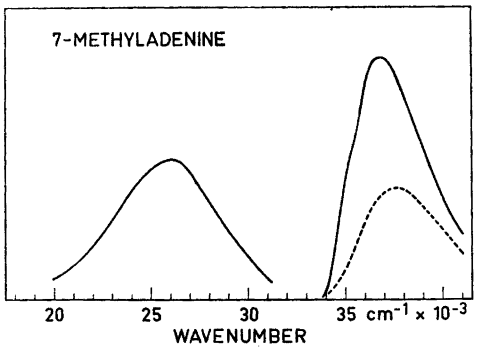

Fig. 5. Fluorescence of 7-methyladenine in aqueous solution at room temperature. $[H_2SO_4] = 10^{-2}$ N. Absorbance at 36 600 $cm^{-1} = 0.30$, light path 1 cm. Same units and meaning of curve symbols as in Fig. 1.

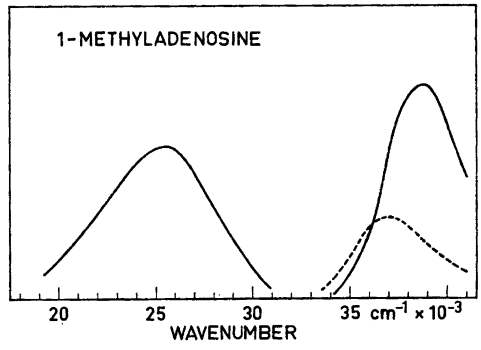

Fig. 6. Fluorescence of 1-methyladenosine in aqueous solution at room temperature. $[H_2SO_4] = 10^{-2}$ N. Absorbance at 38 600 $cm^{-1} = 0.22$, light path 1 cm. Same units and meaning of curve symbols as in Fig. 1.

Effects of pH on the fluorescence. The nonmethylated adenine derivatives have been investigated in this respect previously.[1] Fig. 7 shows that the fluorescence of 7-methyladenine depends on the addition of acid with a pK_a of about 4.2. This pK_a agreed well with the value derived from the change

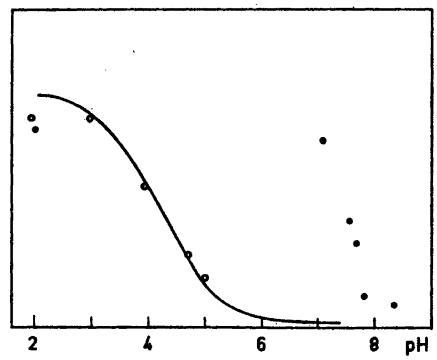

Fig. 7. Effects of pH on the fluorescence of 7-methyladenine and 1-methyladenosine in aqueous solutions.

The solid curve represents the change of the absorbance of 7-methyladenine at 37 000 cm^{-1} as the pH is varied. Below pH 5 pH was adjusted with sulfuric acid, above pH 5 with phosphate buffer and sulfuric acid. Open circles display the influence of pH on the fluorescence intensity of 7-methyladenine. Sulfuric acid was used to adjust pH. The pK_a appears to be near 4.2.

The filled circles show the fluorescence intensity of 1-methyladenosine in 10^{-2} M "Tris" with pH adjusted with sulfuric acid. The effect of increasing the pH above 7 was not reversible, and the fluorescence of a solution with pH about 7.5 disappeared completely after 1 h at room temperature. A chemical degradation is thus superposed on the primary effect of pH, and the pK_a cannot be accurately determined. The absorption spectrum did not change perceptibly between pH 6 and 8.

of the absorption spectrum with pH. No such agreement could be observed with 1-methyladenosine since the absorption spectrum did not change perceptibly in the pH range where the fluorescence varied with pH. Furthermore, the effect of pH on the fluorescence of 1-methyladenosine was not reversible. Probably a secondary chemical degradation follows the loss of a proton.[5] The pK_a appears to be between 7.5 and 8.

Fluorescence quantum efficiencies. Solvent effects. Table 1 shows that the quantum yields dealt with in this paper are very low, *i.e.* in most cases 1 %

Table 1. Fluorescence quantum yields at room temperature. The quantum yields were determined with quinine in 0.5 N sulfuric acid as standard.[19] The approximate fluorescence quantum yields of the fluorescent tautomers have been estimated on the basis of the fraction of the total absorbance at the wavenumber of the exciting light, 37 000 cm^{-1}, which appeared to be attributable to these tautomers. Pyrimidine was investigated in neutral solutions, all the other compounds in 10^{-2} N sulfuric acid.

Compound	Fluorescence quantum yield		
	Aqueous solution	Methanol:water 10:1 (v/v)	Estimated yield of fluorescent tautomer in methanol:water 10:1 (v/v)
Pyrimidine	0.005	0.001	
Purine	0.005	<0.0005	
Adenine	0.0018	0.0023	0.003
Adenosine	0.0008	0.0008	0.0017
Adenosine-5′-monophosphate	0.0015	0.0013	0.0026
Adenosine-5′-diphosphate	0.0038	0.0038	0.0076
Adenosine-5′-triphosphate	0.0046	0.0145	0.030
7-Methyladenine	0.0011	0.0018	0.004
1-Methyladenosine	0.0014	0.0029	0.006

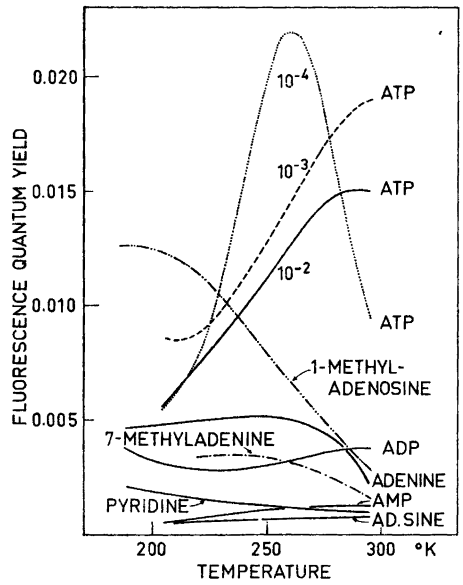

Fig. 8. Effects of cooling on the fluorescence in methanol:water 10:1 (v/v). [H_2SO_4] was 10^{-2} N except in the case of pyrimidine where no acid was added. ATP was also investigated in 10^{-3} N and 10^{-4} N sulfuric acid, as indicated in the figure.

or lower. The addition of methanol seriously quenched the fluorescence of purine and pyrimidine at room temperature (Table 1). The fluorescence intensities of the other compounds were either not greatly influenced or somewhat augmented, the latter effect being notable in the case of ATP.

Fluorescence quantum efficiencies. Effects of cooling. Fig. 8 illustrates that no drastic increase of the fluorescence accompanied cooling down to 200°K. Cooling even decreased the fluorescence of ATP, the details of this effect being dependent on the concentration of sulfuric acid.

DISCUSSION

Excitation spectra and tautomerism. It is generally accepted that large discrepancies between the absorption spectrum and the fluorescence excitation spectrum of a fluorescent solution show that more than one light absorbing solute is present. The disagreements between the two types of spectra described above may thus be due to the presence of impurities, to the formation of molecular complexes, or to the existence of the solute in more than one tautomeric form.

Neither the absorption spectra nor the fluorescence properties of our solutions are likely to have been influenced significantly by impurities. The absorption spectra were in agreement with the data in the literature, and no trace of light absorbing impurities could be seen under UV-illumination of the paper after chromatographic separations. Except for 1-methyladenosine the authenticity of the fluorescence has been confirmed by the observation

that variations of pH influence the fluorescence as could be expected on the basis of the known values of pK_a for the fluorescent compounds.[1]

The formation of dimers and polymers in acid solutions is highly improbable in the case of the adenine derivatives, because the molecules carry a positive charge on or near the purine ring and thus repel approaching molecules of the same kind. The low concentrations of our solutions and the linear dependence of the fluorescence intensity on the concentration constitute further evidence against the significance of molecular complexes. We conclude that the remaining possibility, tautomerism, must be the explanation for the spectral discrepancies observed.

The spectra of protonated purine (Fig. 1) do not give conclusive evidence for or against tautomerism. The differences between the absorption spectra and fluorescence excitation spectra of protonated adenine, adenosine, and the adenosine-5′-phosphates, on the contrary, go far beyond possible experimental errors, and thus constitute strong evidence for the existence of these compounds in one fluorescent and one nonfluorescent tautomeric form. It should be appreciated, though, that this may be an oversimplification, since more than two tautomers may exist, and since the tautomers may fluoresce with different quantum yields rather than be fluorescent or completely devoid of fluorescence. Apparently the fluorescent tautomer is responsible for a smaller fraction of the total absorption of light in the solutions of the adenosine derivatives than in the case of adenine (Figs. 2—4).

The fluorescence of 7-methyladenine (Fig. 5) revealed that fixation of a methyl group on one of the ring nitrogens does not eliminate tautomerism involving remaining protons and heteroatomic free electron pairs. 1-Methyladenosine demonstrated the same phenomenon, although the authenticity of its fluorescence remains to be established. (Figs. 6 and 7).

Structure of the fluorescent and nonfluorescent tautomers. The interpretation of our data is based on the universally accepted principle that the excitation spectrum of a fluorescent tautomer coincides at least approximately with its absorption spectrum.[15] This principle cannot, however, be applied without reservations. The absorption spectra recorded in this work probably are all mixtures of the absorption spectra of more than one tautomer. One cannot expect, therefore, that an excitation spectrum, which is probably due to a single tautomer, can coincide completely with any such recorded resultant absorption spectrum.

The second assumption required in the discussion of the tautomeric structures is that the attachment of a methyl group instead of a hydrogen atom to a nitrogen leaves the fluorescence and absorption spectra almost undisturbed, although the fluorescence quantum yields may be changed. This principle is the rationale for the use of adenine derivatives methylated in known positions to find out the distribution of protons in the nonmethylated compounds in acid solutions. The validity of the structure proposed below for the fluorescent tautomer of protonated, kationic adenine is entirely dependent on the soundness of this second assumption.

Our data shows that protonation of N-1 does occur in adenine in acid solution, and that this protonation yields a tautomer or a group of tautomers which is nonfluorescent. The evidence for this is that 1-methyladenine was

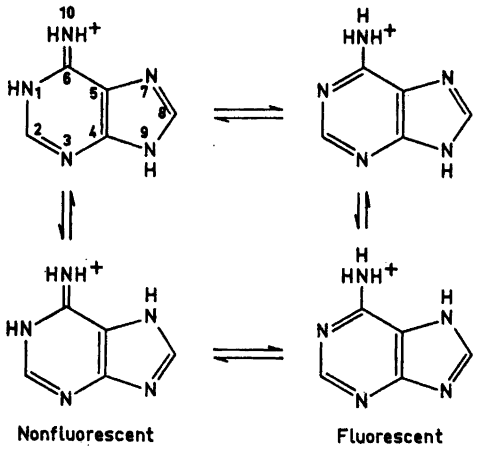

Fig. 9. Proposed tautomeric equilibria of katonic, protonated adenine in solution. The lower right species is probably responsible for the fluorescence. The upper left tautomer must exist in solution because it has been shown to crystallize with Cl^-.[1] No definite evidence has been obtained for or against the existence of the other two tautomers, although the present work suggests that the lower left tautomer is the dominating nonfluorescent tautomer.

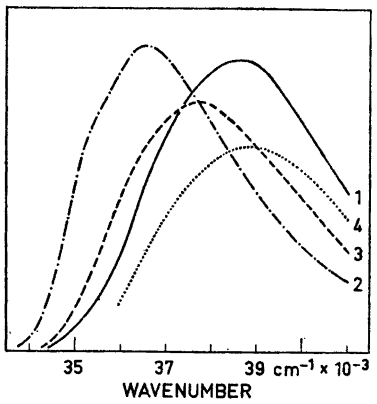

Fig. 10. Absorption spectra of 1-methyladenine (1) and 3-methyladenine (2) compared to estimated absorption spectra of the fluorescent (3) and the nonfluorescent (4) tautomer of protonated adenine. All spectra were obtained in aqueous solutions with 10^{-2} N sulfuric acid.

nonfluorescent and that its absorption spectrum was very similar to the absorption spectrum of the nonfluorescent tautomer of protonated adenine (Figs. 2 and 10). In contrast to this similarity the absorption spectra of 3-methyl- and 7-methyladenine were significantly different from the spectrum of the nonfluorescent tautomer of protonated adenine (Figs. 5 and 10).

The fluorescence of 1-methyladenosine indicates that if N-1 and N-9 are simultaneously engaged in bonds outside the ring, some fluorescence will be observed. This suggests that the nonfluorescent tautomer of adenine in acid solution, which is protonated at N-1, probably has another hydrogen at N-7 rather than at N-9. (Fig. 9, lower left).

The fluorescence excitation spectra of 7-methyladenine (Fig. 5) and protonated adenine (Fig. 2) were almost identical, having maxima near 37 600 cm^{-1}. This suggests that the fluorescence of adenine at low pH is due to a tautomer carrying a proton at N-7. This tautomer probably has no proton at N-1, since it was shown above that such a structure should apply to the nonfluorescent tautomer. N-3 is probably also non-protonated in the fluorescent tautomer, because 3,7-dimethyladenine was devoid of fluorescence.

Another argument confirms that N-1 and N-3 both retain their lone electron pair in the fluorescent tautomer of protonated adenine: If methylation or protonation of N-7 lead to the bonding of a proton to N-1 and/or to N-3,

with the appearance of fluorescence, one would expect, conversely, that the methylation of N-1 or N-3 would yield an entirely equivalent structure except for the interchange of a hydrogen and a methyl group, with fluorescence as a necessary consequence according to the second assumption above. No such fluorescence of 1-methyl- or 3-methyladenine was detected.

These considerations show that there must be either 3 protons in the amino group (Fig. 9, lower right) of the fluorescent tautomer of kationic, protonated adenine, or 2 protons in the amino group and 1 proton at N-9. Since there is a proton at N-7, the latter structure implies that N-7 and N-9 are simultaneously engaged in chemical bonds outside the ring. This is not a likely structural feature according to quantum mechanical calculations [6] and proton magnetic resonance studies.[5] We conclude that the structure shown in the lower right formula of Fig. 9 is the most probable one for the fluorescent tautomer of protonated, kationic adenine, although it cannot be regarded as conclusively demonstrated. This tautomeric structure implies that the amino group is an alternative basic site in adenine.

The proposed structure for the fluorescent tautomer of adenine obviously does not apply to adenosine in acid solutions. First, N-9 in adenosine is engaged in the bond to ribose, and N-7 is then probably not protonated.[5,6] Secondly, in contrast to 1-methyladenine the 1-methyladenosine was fluorescent. The fluorescence excitation spectra of 1-methyladenosine and of protonated adenosine were very similar, both having maxima near 37 000 cm^{-1}: The absorption spectra of the nonfluorescent tautomers (maxima near 39 000 cm^{-1}) were also almost identical in the two cases. Protonation of N-1 thus appears to explain all observations of adenosine at low pH.

The tautomerism of 1-methyladenosine shows that the tautomerism of protonated adenosine is not necessarily due to the occurrence of a tautomer without a proton at N-1, but may alternatively be due to the displacement of a proton from the amino group to a lone electron pair on N-3 or N-7. It is likely, however, that if the amino group is a site of protonation in adenine, it functions as a basic site in adenosine too. Our data do not contain direct evidence for or against the existence of the corresponding tautomeric adenosine with 3 hydrogens bonded to the amino nitrogen.

Our results are not incompatible with the existence of small amounts of the *a priori* probable tautomers protonated at N-3 in acid solutions. All that can be said is that such a tautomer probably does not contribute to the fluorescence of protonated adenine.

It has been claimed that the protonation of adenine and adenosine in acid solutions takes place at the exocyclic amino group rather than at N-1.[16,17] This conclusion was based on the observation that the reaction of formaldehyde with the amino group releases protons (and depresses pH) provided the reaction proceeds at a pH near the acid pK_a of adenine or adenosine. The reaction apparently consumes the base in the equilibrium

$$-NH_2 + H^+ \rightleftharpoons -NH_3^+$$

An alternative interpretation of the pH depression might be that formaldehyde attached to the amino group decreases somewhat the proton affinity of N-1. The formaldehyde reaction consequently does not prove that protonation

occurs at the amino group. It is pertinent to note, however, that the tautomerism formulated in Fig. 9, which implies that the amino group and the N-1 of the ring are alternative basic sites, is able to explain why adenine derivatives sometimes behave as if N-1 were the site of protonation and sometimes as if N-10 were the basic site.

Fluorescence quantum efficiencies. Effects of solvent and temperature. Table 1 and Fig. 8 indicate that the fluorescent tautomers do not attain fluorescence quantum yields in excess of a few per cent at any temperature above 200°K. The literature contains evidence that their fluorescence is equally weak at 77°K.[18]

The curves in Fig. 8 probably do not display exactly the true effects of temperature on the fluorescence yields of the fluorescent tautomers, because cooling may well shift the tautomeric equilibria and thereby alter the concentration of the fluorescent solute. Another complicating effect is exhibited by ATP, *viz.* the strong influence of cooling on the relationship between fluorescence and the concentration of sulfuric acid. The basicity of ATP appears to increase at low temperatures since less acid was required to produce maximal fluorescence at 260°K than at 293°K. Such increases of pK_a values have also been found at 77°K.[18] Higher concentrations of sulfuric acid quenched the fluorescence of ATP, this effect being also dependent on the temperature (Fig. 8).

Temperature effects and types of lowest excited singlet states. The weak fluorescence of pyrimidine [10,11] and pyridazine [11] at room temperature demonstrates that such emissions may well originate from lowest excited singlet states of $n \rightarrow \pi^*$ promotion type. The observation that cooling was unable to increase significantly the fluorescence yields of the adenine derivatives in acid solutions, indicates that these compounds have intrinsically weakly fluorescent n,π^* lowest excited singlet states, rather than intrinsically strongly fluorescent π,π^* singlet states which have been strongly quenched by thermally activated processes. Pyrimidine, which has a lowest excited state of n,π^* type, demonstrated a similar absence of fluorescence increase under the influence of cooling. This suggests that the adenine derivatives represent a new class of compounds which emit some fluorescence light in spite of possessing lowest excited singlet states of n,π^* nature.

Acknowledgements. It is a pleasure to thank Mr. P. D. Lawley for helpful suggestions and the provision of methylated derivatives of adenine.

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