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On the Luminescence Properties of Some Purines and Pyrimidines

A Study by Fluorescence Spectrophotometry of the Sites of Protonation and of the Types of Lowest Excited Singlet States

HANS CHR. BÖRRESEN

Institute of Clinical Biochemistry, Rikshospitalet, University of Oslo, and Norwegian Defence Research Establishment, Kjeller, Norway

The fluorescence of some purines and pyrimidines including adenine and guanine nucleotides has been observed in aqueous solutions at room temperature. The effects of pH are reported in detail. The protolytic reactions are usually found to activate or deactivate the fluorescence, while in some cases only a displacement of the emission maximum is observed. The authenticity of the fluorescence is discussed, on the basis of fluorescence activation spectra and of the values of pK_a deduced from the dependence of the fluorescence intensity on pH. An attempt is made to formulate the mechanism of the fluorescence activating effect of some substituents and protolytic reactions in terms of interchange of the energetic levels of the lowest excited singlet states of $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ promotion type. In the light of this interpretation we consider the sites of protonation of purine nucleotides in acid media, as well as the electronic structure of the excited purine nucleotides at physiological pH.

Nucleotides participate in several biological processes which are sensitive to UV and ionizing radiations. Ultraviolet light, as well as a considerable fraction of the energy absorbed from ionizing radiation, is known to be dissipated as electronic excitations. From this point of view the properties of the electronically excited nucleotides should be of interest both to photobiology and to radiobiology.

The reports in the literature on the luminescence of nucleotides and of other derivatives of purine and pyrimidine are partly conflicting [1–8].

In the present paper the fluorescence at room temperature is reported for aqueous solutions of purine, pyrimidine and some of their derivatives *.

* *Abbreviations.*

AMP Adenosine-5′-phosphate
ADP Adenosine-5′-diphosphate
ATP Adenosine-5′-triphosphate
GMP Guanosine-5′-phosphate
GDP Guanosine-5′-diphosphate
GTP Guanosine-5′-triphosphate

Wavelengths of fluorescence maxima and approximate fluorescence quantum efficiencies are given. The effects of pH are presented in detail. The influence of substituents and of modifications in the aromatic nuclei themselves are analyzed. On the basis of these informations we discuss the reliability of the results, the types of excited states involved, and the loci of the protolytic reactions.

EXPERIMENTAL

Materials. Quinine hydrogen sulphate was obtained from The British Drug Houses Ltd. Purine, pyrimidine and their derivatives were purchased from Nutritional Biochemicals Corp., with the following exceptions: GDP and GTP were obtained both from Mann Research Laboratories Inc. and from Sigma Chemical Company. Adenine was a product of Hoffmann-La Roche & Co.

Procedure. A fluorescence spectrophotometer, Zeiss ZFM 4 C, was used. This was equipped with a xenon lamp (Osram XBO 450 W), two quartz prism monochromators and the RCA IP28 multiplier phototube.

The spectral intensity distribution of the xenon lamp was measured with a thermopile at the exit slit of the first monochromator. The spectral sensitivity curve of the recorder assembly was obtained by comparing its response with that of the thermopile. The latter measurements were performed both with the xenon lamp and with an incandescent lamp as light source. Wavelength band-width curves were used as supplied with the instrument. The transmittance of the monochromators was taken as constant from 290 mμ to 650 mμ as stated by the manufacturers.

The excitation spectrum of pyrimidine in diethyl ether was measured in a solution with considerable extinction coefficient ($E_{240} = 0.5$). The effective quantum intensity distribution of the exciting light was therefore obtained in the following way: A tiny amount of the strongly fluorescent benziminazole (giving rise to insignificant additional absorption) was added to the solution of pyrimidine in ether. The apparent excitation spectrum of the benziminazole was measured. The quantum intensity distribution of the radiation was then computed on the assumption that the true excitation spectrum of benziminazole follows closely the absorption spectrum.

Approximate fluorescence quantum efficiencies were obtained relative to quinine hydrogen sulphate in 0.5 N H_2SO_4, the quantum yield of which is 0.55 [9]. The corrected emission spectra were compared after numerical integration.

Aqueous solutions were made up from doubly distilled water saturated with air. The concentrations used were in the range 10^{-5} M to 10^{-4} M. It was found necessary to remove fluorescent impurities from the diethyl ether by distillation.

When the pH dependence of the fluorescence was studied, the solutions were excited at an isosbestic point or at another wavelength where the absorbancy did not vary appreciably with pH. The pH was adjusted with KOH and H_2SO_4, and measured with reference to standard pH solutions [10] with a glass electrode instrument.

RESULTS AND DISCUSSION

The presentation of the results and the discussion are based on the assumption that the luminescence we have observed is in all cases a fluorescence in the sense of singlet-singlet emission. The long lived phosphorescence (triplet-singlet emission) is supposed to be completely quenched in liquid solution at room temperature [11].

Figs. 1, 2, 3, and 4 show that the addition of H_2SO_4 or KOH often activates or deactivates the fluorescence of the compounds tested. Since the wavelength of the exciting light was chosen in such a way that the fraction absorbed did not depend appreciably on pH, an increase of the fluorescence reflects a rising quantum yield rather than an increasing absorbancy.

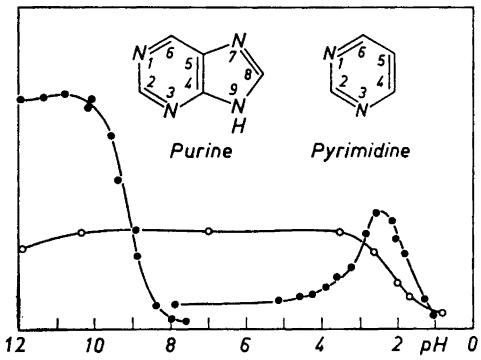

Fig. 1. Fluorescence intensity *versus* pH. Aqueous solutions. ● Purine. O Pyrimidine.

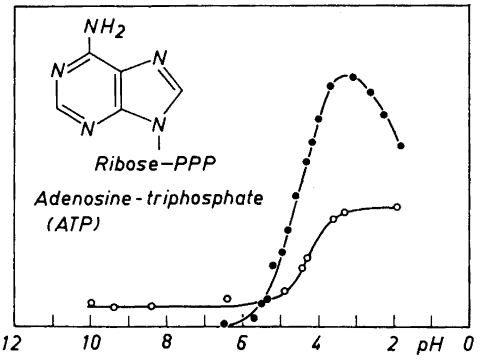

Fig. 2. Fluorescence intensity *versus* pH. Aqueous solutions. ● ATP. O Adenine.

The demonstrated effects of H_2SO_4 and of KOH on the intensity of light emission may be due both to collisional quenching by the added ions and to the addition or removal of a proton on the fluorescent solute. Both these mechanisms may conceivably lead to the familiar sigmoid dissociation curve. Therefore the following criterion was used to separate the effect of pH from the possible quenching by K^+ and SO_4^{2-} ions: Only the influence of pH should be completely reversible when KOH is added to an acid solution or H_2SO_4 to an alkaline solution, since in this way the pH is altered while SO_4^{2-} and K^+, respectively, are kept constant. AMP, ADP and ATP produced definite evidence of quenching by SO_4^{2-} according to this test (Fig. 2). All other effects

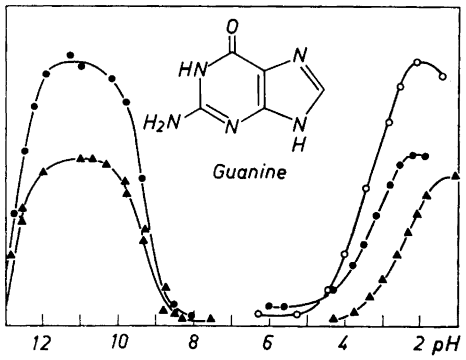

Fig. 3. Fluorescence intensity *versus* pH. Aqueous solutions. ● Guanine. ▲ GMP. O GTP.

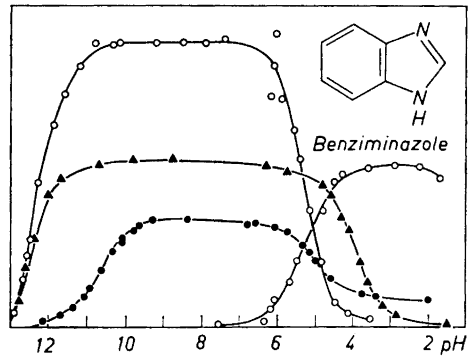

Fig. 4. Fluorescence intensity *versus* pH. Aqueous solutions. ● 2,6-Diamino purine. O Benziminazole. ▲ 2-Amino-4-methyl pyrimidine. The emission maximum of benziminazole is shifted from 290 mμ to 360 mμ when a net positive charge is acquired. The two curves of benziminazole refer to the intensity measured at these two wavelengths.

of KOH and H_2SO_4 dealt with in this paper are predominantly or exclusively due to pH.

The authenticity of the fluorescence was confirmed by the fluorescence activation spectra of all the compounds listed in Table 1 (see example in Fig. 5). The over-all correspondence found between the pK_a of fluorescence activation and the pK_a reported from titration data[10,12,13] also justifies the conclusion that impurities are not responsible for any significant part of the emitted light (Table 1).

In the cases of purine, AMP, ADP, and ATP (Figs. 1 and 2, Table 1) the pK_a deduced from the increase of fluorescence when acid is added to the solution is significantly higher than the true pK_a. A study of the diagrams reveals that as pH is lowered beyond the value of the true pK_a, some new factors decrease the fluorescence intensity. Consequently the curves do not reach the height which they would have attained if only the activating process had been in operation. Clearly this displaces the apparent pK_a towards neutrality.

Table 1. Summary of the essential fluorescence properties obtained. Values of pK_a are quoted from Refs.[10,12,13]. The wavelengths of the emission maxima refer to fluorescence spectra with number of quanta per unit wavelength interval as ordinate.

Compound	pK_a of Fluorescence activation	pK_a Reported in the literature	Maximal fluorescence quantum efficiency	Fluorescence emission maximum, mμ
Adenine	4.2	4.15 9.8	0.003	380
Adenosine	3.7	3.63	0.003	390
AMP	3.95	3.74	0.004	400
ADP	4.3	3.95	0.006	390
ATP	4.6	4.0	0.011	400
2-Amino-4-methyl pyrimidine	4.0 12.5	4.15	0.062	350
Benziminazole	5.3 12.4	5.5 12.3	0.10 (pH 2) 0.16 (pH 9)	360 (pH 2) 290 (pH 9)
2,6-Diamino-purine	5.05 10.7	5.09 10.77	0.022 (pH 8)	340 (pH 2) 350 (pH 9)
Guanine	3.4 9.3 12.7	3.3 9.2 12.3	0.005 (pH 2) 0.024 (pH 10.7)	355 (pH 2) 340 (pH 11)
Guanine deoxy-riboside	2.6		0.015	380
Guanosine	2.2	2.2	0.007	390
G-3′-P and G-2′-P (Mixture)	2.3	2.3	0.017	390
GMP	2.5 9.4 12.7	2.4 9.4	0.008 (pH 1) 0.008 (pH 10.6)	380 (pH 1) 340 (pH 10.8)
GDP	3.1	2.9 9.6	0.018	385
GTP	3.4	3.3 9.3	0.010	380
Purine	3.2 9.2	2.39 8.93	0.008 (pH 2.46) 0.0018 (pH 8) 0.045 (pH 10.6)	400 (pH 2) 380 (pH 5.2) 370 (pH 11)
Pyrimidine	2.0	1.3	0.007 (Water) 0.007 (Ether)	360 (Water) 350 (Ether)

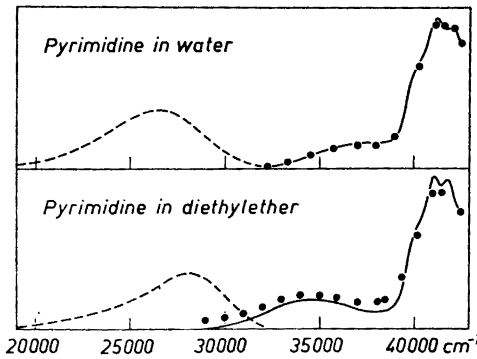

Fig. 5. Optical properties of pyrimidine in diethyl ether and in water.

———— Absorption spectra. Ordinate: Extinction coefficient. The $n \rightarrow \pi$ * absorption maximum is at 35 000 cm^{-1} in ether, but is shifted to 39 000 cm^{-1} in water.
------ Emisson spectra. Ordinate: Quanta per unit frequency interval.
● Relative fluorescence intensity per quantum of incident light, *i.e.* fluorescence excitation spectrum.

The discrepancy seen with pyrimidine is more difficult to explain. The p*K*a deduced from Fig. 1 is about 2.0. This value is at least 0.6 units too high *. Quenching by SO_4^{2-} is of minor importance. The emission does not seem to be due to impurities, since the fluorescence excitation spectra follow closely the absorption spectra of pyrimidine. Fig. 5 shows also that the characteristic shift to higher frequency of the $n \rightarrow \pi$ * absorption is faithfully reproduced by the excitation spectrum when diethyl ether is replaced by water as solvent. If the excited molecule is slightly more basic than the nonexcited species, the apparent value 2.0 for the pK_a would be explained. The frequency of the fluorescence maximum is about 28 000 cm^{-1} in ether (Fig. 5). As predicted [14] it is slightly lower in water, 27 000 cm^{-1}. These maxima are about 4000 cm^{-1} higher in wave number than the phosphorescence maximum [15] of pyrimidine. This is in accordance with our assumption that only singlet states are responsible for the light emission in our experiments. The lowest excited triplet state of the protonated pyrimidine is definitely more acidic than the non excited molecule [14]. We cannot avoid the conclusion that apparently the opposite is true for the lowest excited singlet.

Our results permit some consideration of the electronic mechanisms by which protolytic reactions and introduction of new atoms and groups into a molecule influence its fluorescence intensity. It is well documented that the emission properties of a molecule are governed by the lowest excited singlet state [8] **. Heterocyclic molecules like the purines, pyrimidines and benzimina-

* By means of the shift in the absorption spectrum we have determined the pK_a to be 1.4. In Ref.[12] the value 1.3 is given.

** Even if another singlet state is immediately attained in the absorptive transition, an internal conversion process transforms the molecule into the energetically lowest singlet state usually before any radiation is emitted.

zole possess both n,π^* and π,π^* excited states.* [8,16-18]. Furthermore, in purine, pyrimidine and their biological derivatives the lowest excited singlet states are known to be of $n\rightarrow\pi^*$ promotion type [8,16] in aqueous solution at physiological pH. The currently held opinion is that molecules with lowest excited singlet state of n,π^* type are usually able to emit phosphorescence only (after triplet state formation), while many aromatic molecules with a lowest π,π^* singlet are fluorescent [8]. The fluorescence of pyrimidine indicates, however, that non-fluorescence of molecules with a lowest n,π^* singlet, is not a strictly valid postulate. In spite of this we tentatively interpret the transformation of a molecule from a non-fluorescent to a fluorescent form as representing the replacement of an n,π^* state by a π,π^* state as the lowest excited singlet. The aim of the following discussion is to see to which extent our results can be understood in the light of this assumption. We therefore ask: How do perturbing agents like substituents and the attachment of protons determine the relative energetic levels of n,π^* and π,π^* excited singlet states?

A proton binds a lone pair of electrons. Thereby it increases greatly the energy needed in the transition which consists in the promotion of one of these electrons into a vacant π^* orbital. If there is no other n,π^* state which takes over the role as lowest excited singlet, a π,π^* configuration will become the lowest one. We propose such a mechanism to account for the fluorescence in acid solutions of purine as well as of adenine, guanine and their biological derivatives (Figs. 1—3). This interpretation is confirmed in the case of the compounds containing adenine by the results of Steele and Szent-Györgyi [3]. They report phosphorescence at liquid nitrogen temperature from the non-protonated molecules only, leading to the conclusion that only the latter possess lowest n,π^* singlet states.

The n,π^* states other than the one which involves the orbital to which the proton is attached through a σ bond, are, on the contrary, expected to be stabilized by the protonation. The $n\rightarrow\pi^*$ transitions are associated with a considerable displacement of negative charge from the localized lone pair orbital to a π^* orbital covering the whole molecule [19]. The positively charged proton may well favour such a redistribution of negative charge. When this second effect of a proton is dominating, the protonated molecule will be non-fluorescent. Purine furnishes an example of this mechanism. When the second proton is added in acid solution below pH 2.5, the n,π^* state which remains is presumably stabilized to such an extent that no excited π,π^* singlet is energetically more favourable. It is likely that the excited purine is responsible for the acquirement of the second positive charge, since purine in the ground state is not known to exhibit this behaviour [13]. The disappearance of the fluorescence

* A π,π^* state is the immediate result of a $\pi\rightarrow\pi^*$ transition in which one electron is lifted from a doubly occupied π orbital to an empty, antibonding π^* orbital. An $n\rightarrow\pi^*$ transition correspondingly involves the promotion of one electron from a nonbonding orbital containing a lone pair of electrons into an empty, antibonding π^* orbital. A prerequisite of the n,π^* state is the presence of atoms like nitrogen, oxygen or sulphur in the molecule. The heteroatom must contribute a p orbital to the system of π orbitals, but still possess a lone pair of electrons. The $n\rightarrow\pi^*$ transition has a comparatively low intensity, and the n,π^* state a correspondingly long intrinsic mean lifetime (in the absence of quenching processes). Hence there is time enough for processes other than fluorescence to prevail.

of 2-amino-4-methyl pyrimidine as the pH is lowered in the acid range (Fig. 4) can be given the same explanation except that it is not necessary to invoke the excited molecule as the proton acceptor.

The loss of a proton must have two effects just opposite to those mentioned above. A lone pair orbital is set free and consequently a new and possibly lowest n,π^* state is introduced. But on the other hand all other $n\rightarrow\pi^*$ transitions will be opposed to the extent that the negative charge is spread out on the entire molecule. The latter effect is similar to the one postulated to account for the shift of $n\rightarrow\pi^*$ transitions to higher frequencies which is brought about by electron-donating substituents like $-NH_2$ and $-Cl$. Again it can be anticipated that fluorescence and non fluorescence may both be the outcome of a protolytic reaction. In the following examples the decisive influence of the type of aromatic nucleus, the net molecular charge, and the nature of the substituents will be shown.

We consider a sequence of molecules with the accepted values of the relevant pK_a mentioned in parenthesis: Purine (8.9), guanine (9.2), adenine (9.8), 2,6-diamino purine (10.7), benziminazole (12.3) and 2-amino-4-methyl pyrimidine (> 12). The lower the value of pK_a, the stronger is the pull exerted by the rest of the molecule on the electrons surrounding the acidic nitrogen. The position of this nitrogen and the detailed shape of the molecule do not concern our argument. When a proton is lost in the pH range of the pK_a given, each of these molecules acquire one net negative charge, but only purine and guanine demonstrate fluorescence. A clue to the explanation is the strong power with which the negative charge left behind at the nitrogen in purine and guanine is attracted to other parts of the molecule, as appears from their pK_a. In the other compounds this charge will continue to reside on the nitrogen to a greater extent. The electron-repellant action of this localized negative charge will favour preferentially the $n\rightarrow\pi^*$ transition, which consists in the displacement of one electron from the negative nitrogen to the rest of the molecule. Hence among the molecules with one negative charge those more likely to possess lowest n,π^* singlet states are found be nonfluorescent.

Fig. 3 shows that GMP behaves like guanine at pH values > 7. Furthermore it is seen that as soon as two negative charges are acquired by deprotonation the light emission is again zero. The π orbitals already possessing one negative net charge have no great affinity for the second one, as shown by the high values of the second pK_a. The situation consequently resembles the one in the four last molecules in the above sequence. The other guanine derivatives were all non fluorescent at pH values > 7. A possible explanation is that the excited molecules are too acidic: An additional proton will be lost as soon as the negatively charged molecule is excited. Thus the guanine derivatives are not as uniform with respect to fluorescence in alkaline media as stated by Udenfriend [6].

The conditions for the fluorescence of electrically neutral molecules can be seen from the same sequence of compounds which was considered above. The argument is analogous. But now there is no question of an electron-repelling charge on a nitrogen in a hypothetical π,π^* state. On the contrary we have to take into account the direct effect of the electron affinity of the π orbital system on the energetic levels of n,π^* excited singlets. The more strongly

the π orbitals attract electrons, the lower will the pK_a be, and the more will the charge displacements of the $n \rightarrow \pi^*$ transitions be favoured. Thus it can be explained that only the first three compounds (with the smallest values of pK_a in the alkaline range) are non fluorescent, whereas the other compounds of the series are strongly fluorescent when electrically neutral.

In general, fluorescence is never seen both before and after the acquirement of a negative charge. This can be understood when it is observed that a high pK_a favours fluorescence before the proton is lost, while a low pK_a is necessary for fluorescence after the proton is lost.

A comparison of purine with 2,6-diamino purine and of pyrimidine with 2-amino-4-methyl pyrimidine serves to discern the effects of amino substituents. They donate electrons by resonance and consequently oppose the $n \rightarrow \pi^*$ transitions [19]. For the same reason the acidity of the purine nucleus is decreased, (pK_a increases from 8.93 to 10.77). In 2-amino-4-methyl pyrimidine the pK_a of about 12.5 must represent the acidity of the substituent itself. Since this pK_a is not found with ordinary titration procedures [12], it may be a property of the excited molecule only. When attached to the 2-carbon of the ring, the amino group displaces the lowest $\pi \rightarrow \pi^*$ absorption maxima towards longer wavelengths [13]. This maximum is 263 mμ in purine, 280 mμ in 2,6-diamino purine, 243 mμ in pyrimidine and 288 mμ in 2-amino-4-methyl pyrimidine. The stabilization of the π,π^* singlets relative to the n,π^* singlets is therefore due to two mechanisms. The strong fluorescence of the amino substituted compounds in the neutral state is thus accounted for. The high values of the pK_a, which are due to the amino groups, explain the non fluorescence of the negatively charged molecules.

6-Amino purine (adenine) is, however, non fluorescent both in neutral and in alkaline solutions. Its $\pi \rightarrow \pi^*$ absorption maximum is 260 mμ. From this observation it follows that the electron donating power of an amino substituent is not sufficient to bring about an inversion of the relative energetic levels of the n,π^* and π,π^* singlets in the neutral molecule. Evidently it is necessary that the $\pi \rightarrow \pi^*$ transition be displaced to lower frequencies as well. When the negatively charged adenine is compared with the fluorescent, negatively charged purine, it appears, however, that the higher pK_a in adenine has favoured sufficiently the $n \rightarrow \pi^*$ transition introduced by the deprotonation.

As to the sites of protonation of adenine, guanine and their derivatives in acid solution, our results point towards the nitrogens within the heterocyclic rings. The addition of a proton to an amino substituent destroys its conjugative power, but it does not rule out any $n \rightarrow \pi$ transition. Studies of the effects of protonations of adenosine and guanosine on their infrared absorption spectra lead to the same conclusion [20]. When these findings are taken into account together with crystal structure analyses [21,22], more exact locations of the protons can be obtained: N-1 in adenine and N-7 in guanine. If these assignments are valid for the other purine derivatives as well, we suggest that the lowest excited singlet states of adenine nucleotides and guanine nucleotides at physiological pH not only are of $n \rightarrow \pi^*$ promotion type, but involve the lone pair orbitals of N-1 and N-7 respectively.

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