Eur Biophys J (1988) 16:131–142

European Biophysics Journal

Synchrotron-excited time-resolved fluorescence spectroscopy of adenosine, protonated adenosine and ^{6}N,^{6}N-dimethyladenosine in aqueous solution at room temperature*

J.-P. Ballini [1,3], M. Daniels [2,3], and P. Vigny [1,3]**

[1] Laboratoire de Physique et de Chimie Biomoléculaire C.N.R.S. (UA198) Institut Curie 11, rue Pierre et Marie Curie, F-75231 Paris Cedex 05, France, and Université Pierre et Marie Curie (Paris VI)
[2] Chemistry Department and Radiation Center, Oregon State University, Corvallis, OR 97331, USA
[3] Laboratoire d'Utilisation du Rayonnement Electromagnétique (L.U.R.E.), C.N.R.S. and Université Paris-Sud, Bât. 209 C, F-91405 Orsay Cedex, France

Received October 14, 1987/Accepted in revised form February 19, 1988

Abstract. The fluorescence behavior of adenosine in neutral solution has been studied by time-resolved spectroscopy using synchrotron excitation and time-correlated single photon counting, and by decay time measurements. Three emissions have been identified and correlated with three excitation spectra. The assignment of these transitions has been made by comparison with similar measurements on ^{6}N,^{6}N-dimethyladenosine (6DMA), and on adenosine in acid solution (ADO H^+). It is proposed that two of the transitions of adenosine which correlate with 6DMA originate from coplanar and orthogonal rotational conformers of the amino group. The other transition, correlating with ADO H^+ may originate either from the ^{3}H-imino tautomer, or from a differently solvated rotational conformer.

Key words: Adenosine, time-resolved fluorescence spectroscopy, fluorescence excitation, decay time of fluorescence, dual fluorescence

Introduction

The fluorescence from adenosine (ADO) and adenosine-5′-monophosphate (AMP) in neutral aqueous solution at room temperature has been little studied despite its obvious importance for understanding excited state behavior and photochemistry of many polymeric nucleic acid species. In contrast, there have been many studies on adenine (Ade) and AMP in acid solution and at low temperature. The origin for such disparate treatment lies not in the intrinsic interest of acidic or low temperature media but is found simply in the fact that the fluorescence quantum efficiency of ADO and AMP at room temperature is so low ($\approx 4 \times 10^{-5}$) as to make detection difficult with conventional apparatus, while in acidic solution ϕ_f is around twenty times greater and at 77 K the increase is by three orders of magnitude.

The advent of brilliant light sources such as the UV laser or the synchrotron has completely changed this situation and now makes possible the extended investigation of very weak emitters such as adenosine. The work reported here has been carried out on the fluorescence lifetime facility which is coupled to the UV/visible port of the ACO synchrotron at LURE, Orsay (France). While it might appear that use of a psec. UV laser would have been to advantage, in retrospect an entire class of measurements which we term 'time-resolved excitation spectra', requiring repeated scanning over a range of 16,000 cm^{-1} of excitation frequency in the UV (from 230 to 357 nm), would be so tedious and unreliable as to be impracticable for an extended piece of research such as this.

The starting point for the present work was our observation (Ballini et al. 1982) of an unexpected 'tail' during the measurement of the decay profile of the fluorescence from adenosine (Fig. 2). The significance of this arises from the fact that from the quantum efficiency and intrinsic lifetime (calculated from the spectra) a fluorescence lifetime of ≈ 1 ps would be anticipated. With the 1.76 ns exciting pulse of the LURE synchrotron, the fluorescence profile should then be that of the exciting pulse. Our observation suggested the presence of some long-lived species and aroused our interest because of a much earlier brief report (Blumberg et al. 1968) of an anomalously long ≈ 4.3 ns lifetime at 77 K. Accordingly we have carried out the extensive series of measurements reported here utilizing variable-wavelength time-correlated single photon

* A partial presentation of this work has been made at the Second Congress of the European Society for Photobiology Padova, Italy, 6–10 September 1987

** To whom offprint requests should be sent

counting detection in conjunction with variable wavelength 1.76 ns pulsed excitation at 13.6 MHz. This technique is particularly suitable for carrying out time-delayed measurements and as well as the conventional time-delayed emission spectra we have relied heavily on the corresponding time-delayed excitation spectra, a much less common procedure, to elucidate the behavior of adenosine at room temperature. With the aim of aiding the interpretation of neutral adenosine we have carried out similar measurements on ^{6}N,^{6}N-dimethyladenosine (6 DMA), ^{6}N-methyladenosine (6 MA), 1-methyladenosine (1 MA), and on protonated-adenosine (ADO H^+).

Experimental

All experiments have been carried out using the pulsed synchrotron excitation source together with gated time-correlated photon counting. The general synchrotron facility at LURE has been completely described earlier (Guyon et al. 1976). The radiation consists of almost gaussian pulses, FWHM = 1.76 ns, at a repetition rate of 13.6 MHz (73 ns between pulses) for single bunch operation. For the time-resolved fluorescence and lifetime work the beam is brought out via a sapphire window, transported to and focused into an SLM 2800 spectrofluorimeter. This set-up has been outlined earlier (Ballini et al. 1982, 1983), and it is only necessary to emphasize the use of a double excitation monochromator with holographic gratings in recording the excitation and emission spectra, and the decay profiles. A measure of the overall sensitivity of the facility may be gained by noting that while most work in fluorescence decays is with systems for which the product of emission quantum yield ($\phi_f \approx 0.1$) and absorbance ($A \approx 0.1$) is $\approx 10^{-2}$, the work we report here and in (Ballini et al. 1982, 1983) is for systems for which $\phi_f A \approx 10^{-4}$. This is only made possible by the high brightness of the synchrotron source (approximately two orders of magnitude higher than a 150 W xenon arc) combined with the low stray light of the optics.

Time-resolved spectroscopy

The arrangements for signal handling which allow us to determine time-resolved spectra is illustrated in Fig. 1 a and b. Figure 1 a shows the more usual (lifetime-mode) operation of the time-amplitude converter (TAC)/pulse-height analyser (PHA) combination. The TAC generates a pulse whose magnitude is linearly related to the time between the start and the stop pulses. The TAC output pulses are then sorted in the PHA and stored in an address which corresponds to the time after excitation at which a photon is detected. For the time-delayed spectra mode (Fig. 1 b) the TAC output is sorted by a two level discriminator (single channel analyser, SCA) which only gives an output when the input falls between its levels. In this way the lower level discriminator (LLD) determines the time delay Δt, and the upper level discriminator (ULD) determines the acceptance time window δt. Output pulses from the SCA are then counted in the analyser now operating in its multi-channel scaling (MCS) mode. Channel advance, and hence counting time/λ interval, is determined by the monochromator digital scan control. Obviously many counts can be lost in this mode, but the selection of the time window is very precise and sharp (0.078 ns/channel) and jitter-free in contrast to most other methods of gating.

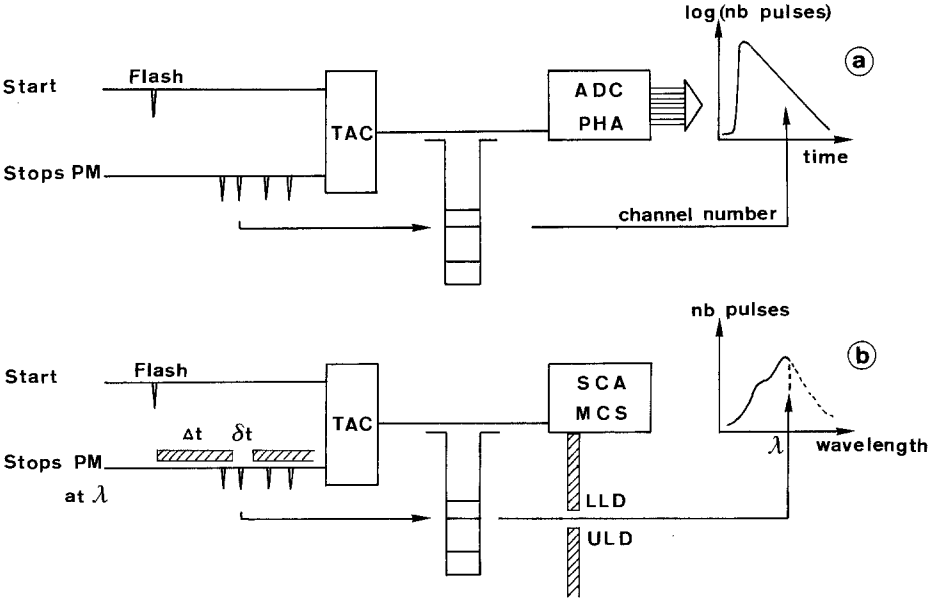


Fig. 1. a Lifetime mode operation of TAC/PHA. **b** Time-delayed spectra mode of operation (SCA/MCS). Another mode of operation is pulse heigh analysis within a time-window (SCA/ADC/PHA). With this mode, it is possible to see which part of the decay time is determined by LLD and ULD, before setting them for **b** mode of operation

Channel resolution over 128 ch. is 2 nm for a scan range from 280 to 536 nm in emission, or over 64 ch. from 220 to 347 nm in excitation. Optical resolution has been either 16 nm or 8 nm, for either excitation or emission spectra, depending on the compound. The dwell times are ≈ 1 s/ch./scan and the times required to collect these spectra typically are 21 min for six scans. No smoothing has been carried out on the results presented here. Correction for the decay of the synchrotron beam is unnecessary during the recording of one spectrum because of the up-down-up-down, scanning sequence and the long lifetime of the beam, while it is taken into account between recordings of sample and buffer. The geometry of the beams within the 1 cm cuvette has been verified in order to correct the emission and the excitation spectra for the absorbance of the solution, using the procedure of Vigny and Duquesne (1974) (OD has always been kept $\leqq 1$). Excitation spectra are corrected for the wavelength variation of exciting beam intensity using solid sodium salicylate as a quantum counter. All other conditions and materials have been described previously (Ballini et al. 1982).

The time-delays and windows used in this work are illustrated in Fig. 2 for the fluorescence from adenosine. The following time-windows have been commonly used.

a) early time-window, 0 → 2.35 ns
b) intermediate time-window, 2.35 → 4.70 ns
c) late time-window, 4.70 → $\leqq$ 36.0 ns.

Most of the time, the intermediate time-window has been used qualitatively to help us assign the species in going from early to late time-window.

Decay kinetics

Protocol for decay-time recording; decays have been measured at excitation and emission wavelengths suggested by the time-delayed spectra. Recording of one decay is always carried out using a sequence of four measurements. (*i*) an exciting pulse profile (preflash) taken from the buffer as scattering solution, at the emitting wavelength of our sample, with a constant geometry of the beams within the cuvette and onto the photomultiplier, and with the same counting rate of events as for the decay profile (obtained by aperturing the exciting beam before the double monochromator). The count rate per excitation flash is very low ($\approx 10^{-4}$ to 10^{-5}), so that no pile-up correction has to be considered. (*ii*) decay profile of our sample. (*iii*) decay profile of the buffer under the same conditions. (*iv*) another exciting pulse profile (postflash) identical with (*i*) in order to verify the stability of the experiment.

Decay-time analysis; we use the same treatment concerning the absorbance of the solution and the decay of the synchrotron beam as for spectra. Then analysis has been carried out on a Vax 780 by re-convolution from the excitation pulse profile assuming a multi-exponential model. Fitting is by non-linear least squares minimisation using the Marquardt algorithm, the goodness of fit being judged graphically by randomness of the plot of the weighted residuals and of the autocorrelation function of the weighted residuals, and by the value of χ^2. Each decay determination always consists of at least two data sets because of pre- and post-flashes, and the computation is carried out a number of times (5 to 10), varying the starting values, in order to cheek the stability of the analysis. Despite all these precautions, because of the extremely low quantum yields of our compounds, some instabilities remain in our analysis. For sets of multicomponent decays for which the same decay constants are anticipated, only the proportion of each varying, global analysis of all the sets leads to a considerable reduction in number of independent parameters (Knutson et al. 1983; Frye et al. 1984). Thus for a two component decay, measured at three wavelengths, global analysis reduces 12 parameters to 8. In our protocol, using pre- and post-flash recording, 24 parameters are reduced to 14. Such global analyses have always been carried out with a range of starting values.

Absorption spectra have been measured with a Cary 118 CX spectrophotometer.

To be sure of the purity of adenosine, liquid chromatography separation has been carried out on a Beckman model 344 HPLC system, using an Altex Ultrasphere-ODS column (25 × 0.46 cm; mean particle size 5 μm) at 35 °C, eluting with a gradient of 2.5% to 12.5% methanol/water at a flow rate of 1.5 ml/min, monitoring by absorbance at 254 nm on a Shimadzu detector. No evidence of more than one peak has been found.

Adenosine (ADO) was obtained from Calbiochem and from Boehringer, ^{6}N,^{6}N-dimethyladenosine (6 DMA) from Sigma, ^{6}N-methyladenosine (6 MA) and 1-methyladenosine (1 MA) from P-L Biochemicals. Solutions were usually prepared in neutral buffer with phosphate 10^{-2} *M*, pH 7.2. The pH of acidic solutions has been adjusted using sulfuric acid and the pH of alkaline buffer was adjusted with sodium carbonate.

Results

A. Adenosine

i) Time-delayed emission spectra. As noted in the Introduction, our first decay profiles such as Fig. 2 indicated that, excited at 275 nm and monitored at 390 nm, most of the emission is much faster than nano-

second, as expected, together with a much smaller proportion of longer-lived component with τ in the nanosecond range. To determine the emission spectrum of the fast component with the minimum of interference from slower-decaying component(s) the emission spectrum has been determined using the early time-window with the results shown in Fig. 3. With an onset

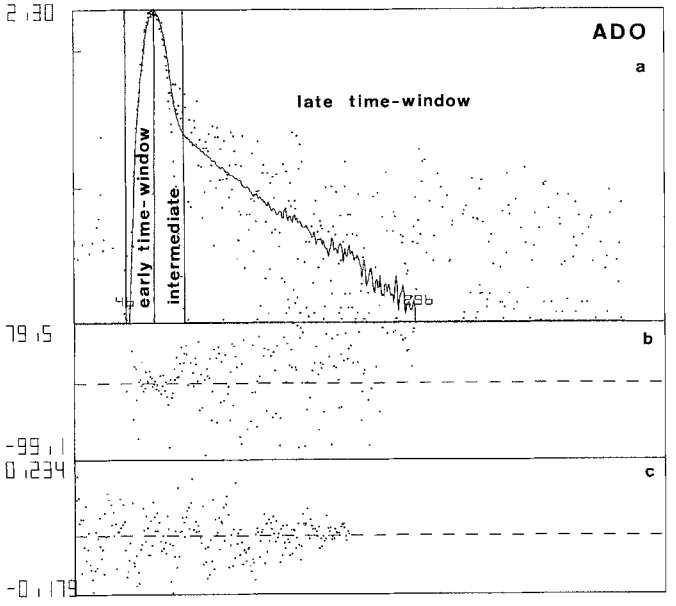


Fig. 2. a *Cross*: Decay profile for neutral adenosine fluorescence excited at 275 nm and monitored at 390 nm. Log (counts) versus 0→511 channel number (time scale: 0→40 ns). *Black dot*: Exciting pulse profile. *Continuous line* is the fitting by two exponentials. Early time window within the leading edge of the excitation. Late time window after the excitation. **b** Weighted residuals. **c** Autocorrelation function of the weighted residuals

$\approx$ 290 nm, $\lambda_{max} \approx 320$ nm and $\lambda_{1/2} \approx 360$ nm, this result is closely similar to that observed for ADO at 77 K in glycol/water (Longworth et al. 1966). No other species is obviously present and we shall refer to this spectrum as originating from the '320' species.

To discriminate against the '320' species and to favor the slower-decaying species responsible for the 'tail' of Fig. 2 is the reason for using the intermediate and late time-windows. In the intermediate time-window the spectrum has strengthened on the long-wavelength side, although the '320' emission is still significant and the maximum remains ≈ 320 to 330 nm. In the late time window (Fig. 3) there is considerable gain of intensity at 360 nm, and beyond to 500 nm while little of the '320' remains. It is important to note that the intensity at 390 nm is very close to that at 360 nm and a slight change in window can lead to a reversed intensity ratio. This suggests that the emissions at 360 nm and 390 nm may originate from different species. To help clarify the number of emitting species and their relationships, excitation spectra have been determined, as well as decay profiles at different wavelengths.

ii) Time-delayed excitation spectra. These are shown in Fig. 3. The '320' species has been monitored not at its maximum but at 340 nm to avoid interference by Raman scattering. For the early time-window an excitation spectrum is obtained which superimposes very nicely with the absorption spectrum of adenosine, peaking at 258 nm. For the intermediate time-window, monitoring the slow emission at 400 nm gives a quite different excitation profile which peaks ≈ 267 nm and extends to wavelengths above 300 nm.

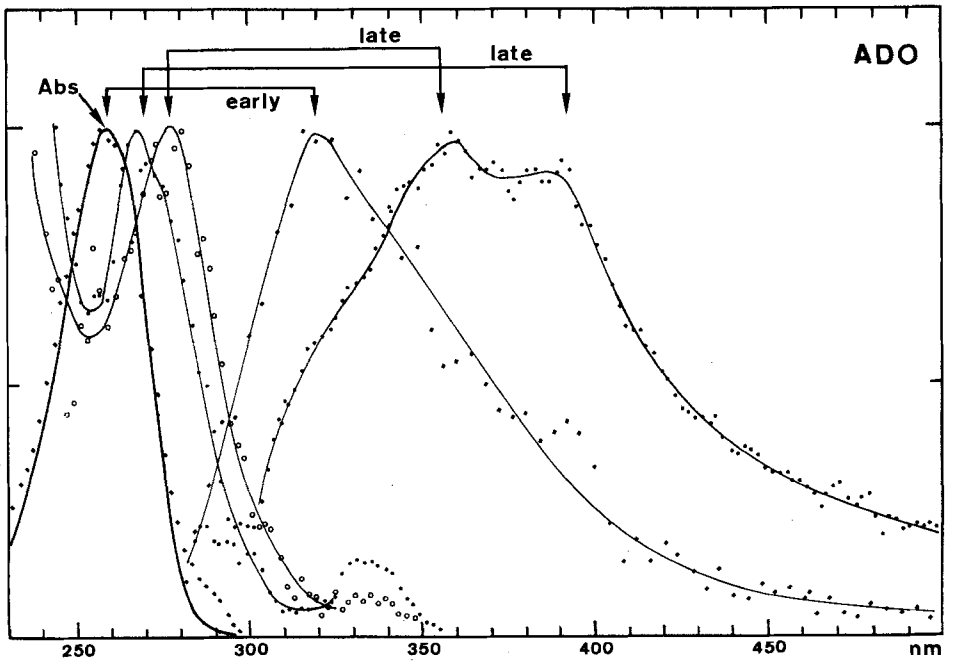


Fig. 3. Time-delayed emission and excitation spectra for neutral adenosine. *Black square*: Early time-window emission ($\lambda_{ex} = 250$ nm), excitation monitored at $\lambda_{em} = 340$ nm. *Black dot*: Late time-window emission ($\lambda_{ex} = 270$ nm), excitation monitored at $\lambda_{em} = 400$ nm. *Circle*: Late time-window excitation monitored at $\lambda_{ex} = 360$ nm. Corresponding emission and excitation spectra are indicated by *double-headed arrows*. Absorption spectrum is *continuous line*

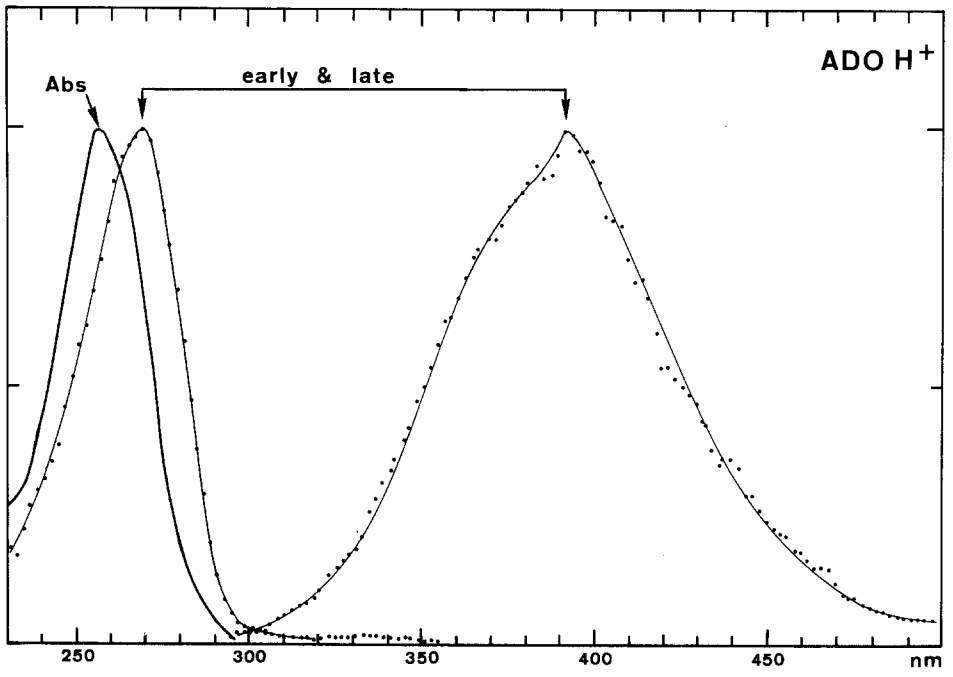


Fig. 4. Time-delayed emission and excitation spectra for acidic adenosine. *Black dot*: Early and late time-window emission (λ_{ex} = 260 nm), excitation monitored at λ_{em} = 390 nm. Corresponding emission and excitation spectra are indicated by *double-headed arrows*. Absorption spectrum is *continuous line*

The late time-window profile monitored at 400 nm peaks $\approx$ 269 nm (Fig. 3) while monitored at 360 nm the peak is broader and extends to $\approx$ 281 nm with a mean of the values around 276 nm. In interpreting data such as this it must be noted that such time-delayed spectra do not represent single species but are the result of the overlapping in wavelength and time of several emitting species which have been partially resolved by appropriate selection of λ_{ex}, λ_{em}, Δt and δt.

It should be noted that an additional small excitation peak is observed around 330–340 nm. Its wavelength position and the time-window used in its recording exclude the possibility that it is Raman scattering. No definite interpretation can be given at the present time.

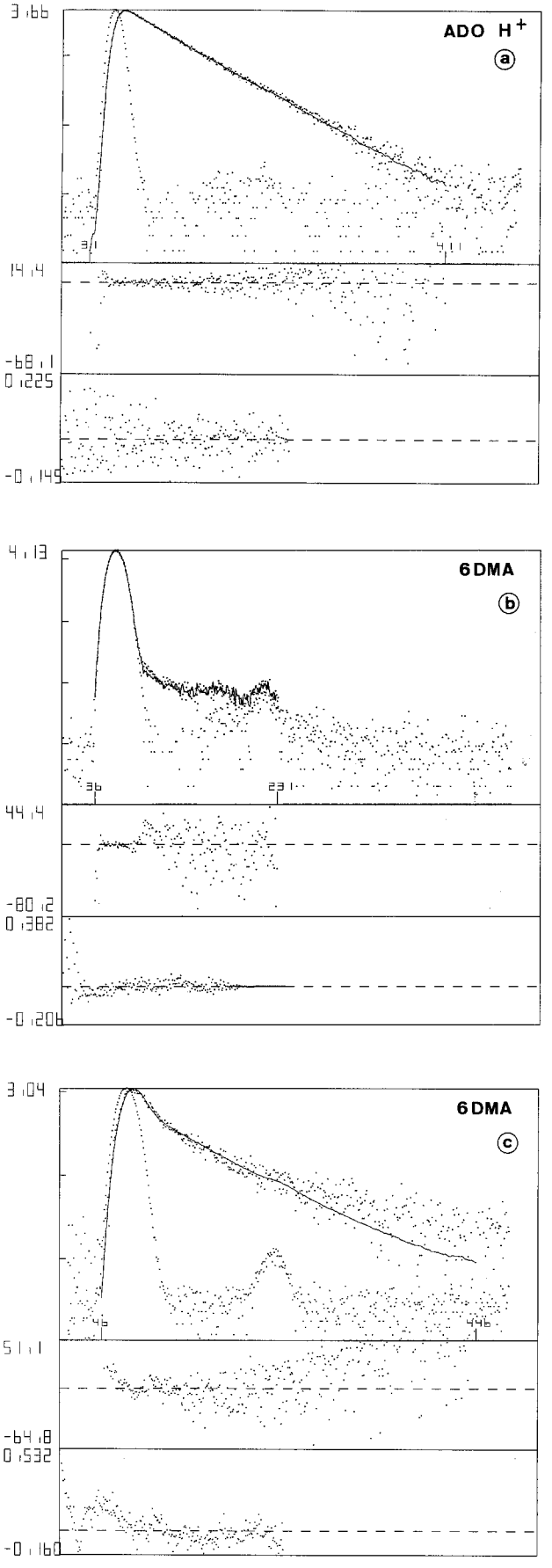
The behavior in the intermediate and late time windows may be interpreted as indicating the existence of two species having lifetimes in the nanosecond range, the first emitting most strongly around 360 nm and having an excitation peak $\approx$ 276 nm, the second emitting primarily at longer wavelengths $\approx$ 390 nm and having an excitation peak $\approx$ 269 nm. Our understanding of this system at this stage may be summarized as follows

	λ_{ex}(max)	λ_{em}(max)	τ
Species I	258 nm	320 nm	$<$100 ps
Species II	$\approx$ 269 nm	$\approx$ 390 nm	$\approx$ 4 ns
Species III	$\approx$ 276 nm	$\approx$ 360 nm	$\approx$ 4 ns

Further evidence has come from investigating the decay profiles corresponding to this classification.

iii) Wavelength-resolved decay profiles. Typical decay profile is shown in Fig. 2. Excitation at 260 nm and monitoring at 320 nm gives mostly (84%) a very fast emission which can only be characterized as having $\tau < 100$ ps. This is expected for adenosine based on the quantum yield and intrinsic lifetime calculated from the entire first absorption band. However there is also a small amount (16%) of a longer-lived component with a lifetime of 3.4 ns and this is probably due to a small spectral overlap from another species which emits maximally at another wavelength. Exciting at 250 nm and monitoring at 330 nm gives essentially the same results (16.5% of a 3.9 ns component), however changing the excitation to 275 nm while still monitoring at 330 nm changes the proportion of longer-lived component to 56% without significantly changing the lifetime (3.7 ns). These results are consistent with the idea that there are two emitting species in this region, a very fast one with an excitation maximum at $\approx$ 260 nm and another with a much longer 3.7 ns lifetime preferentially excited between 270 nm and 280 nm. Exciting at 275 nm and monitoring at 390 nm or 350 nm gives 58% or 55% of a nanosecond component with a lifetime of 4.1 ns (global value) and this is also consistent with time-delayed spectra suggesting there are two species resulting from 275 nm excitation.

While the emission from neutral adenosine peaking at 320 nm and decaying much faster than the exciting pulse can be immediately assigned as the 'normal' or anticipated fluorescence from adenosine on the basis of the agreement of its excitation spectrum with the absorption spectrum of adenosine, the nature of the species responsible for the longer lived emissions in the



range 350–400 nm is not obvious. From the similarity of this emission with the fluorescence from adenosine in acidic solution ($\lambda_{max} \approx 390$ nm) it occurred to us that we may be observing emission from protonated adenosine even in neutral solution. To provide a better basis for such an assignment we have investigated in detail the fluorescence behavior of adenosine in acid solution in the same manner as reported above for neutral solution.

B. Adenosine in acidic solution

i) Time-delayed emission spectra. For all time-windows the spectra are remarkably similar all having a peak ≈ 390 nm, the major difference between the early and late windows being a small red shift of the slopes of the curves. Figure 4 is a typical spectrum.

ii) Time-delayed excitation spectra. In view of the similarity of the emission spectra it is not surprising that the excitation spectra are almost identical for all time windows. An example is in Fig. 4. The outstanding feature is the excitation maximum at 269 nm, compared with the absorption maximum at 256 nm. To examine the possibility that a weak fast emission similar to species I might be hidden under the high energy slope of the major emission the excitation spectrum in the early time-window was repeated, monitoring at 340 nm. No change in excitation peak was observed. Consequently from time resolved spectra protonated adenosine is well described as a single component in emission. However the excitation spectrum is clearly different from the absorption spectrum (Fig. 4), implying the existence of at least two absorbing species.

iii) Decay profile. Measured at 350 nm or at 390 nm the decay profile (Fig. 5a) cannot be analysed satisfactory as a single component. Excited at 270 nm, two component analysis at 350 nm gives 95% of a 4.5 ns and 98.6% at 390 nm. The proportion of the fast component is so low that a considerable uncertainty attaches to its lifetime which can only be given as <0.5 ns.

The difference between the excitation spectrum and the absorption spectrum of adenosine in acid solution recalled the extensive early work of Börresen (1967) on tautomerism and suggested this might be involved in the behavior of adenosine in neutral solution. To provide information which might aid us in evaluating this

Fig. 5. Decay profiles of a) acidic adenosine. $\lambda_{ex} = 270$ nm, $\lambda_{em} = 390$ nm, b) 6DMA $\lambda_{ex} = 270$ nm, $\lambda_{em} = 360$ nm, c) 6DMA $\lambda_{ex} = 315$ nm, $\lambda_{em} = 400$ nm. log(counts) versus 0→511 channel number (time scale: 0→40 ns). *Solid line*: Weighted residuals and auto-correlation function of the weighted residuals are calculated for two-components analysis

possibility we have examined the fluorescence behavior in neutral solution of methylated derivatives, for which tautomerism (Fig. 6) is not possible or is considerably restricted.

C. $^{6}N,^{6}N$-dimethyladenosine (6 DMA)

Excited at 260 nm the early time-window gives a broad emission spectrum $\lambda_{max} \approx 355$ nm (Fig. 7). At first sight this seems considerably red-shifted from adenosine but the absorption spectrum is also red-shifted ($\lambda_{max} \approx 275$ nm) so that the Stoke's shift, 8,100 cm^{-1} is similar to that for adenosine, 7,700 cm^{-1} (see also Table 1). The excitation spectrum corresponding to this emission superimposes on the absorption spectrum (Fig. 7). Although 6 DMA can only exist in one tautomeric form, the late-time window emission spectrum excited at 315 nm is quite different from the early window. A similarity exists with the late time-window of adenosine but the spectrum is more than an order of magnitude weaker and has a half-width of 6,000 cm^{-1} compared with 8,000 cm^{-1} for adenosine. Consequently, although adenosine shows two emission components in this time-window, 6 DMA shows only one. The excitation spectrum corresponding to this late emission is completely resolved from the absorption spectrum and peaks at 315 nm with a half width of 4,700 cm^{-1}. Consistent with the behavior of this system as two independent excitation/emission components, the decay kinetics are bi-exponential when excited at 270 nm and monitored at 360 nm, with >90% of fast component (<100 ps) (Fig. 5b), and with ≈90% of slow component when excited at 315 nm and monitored at 400 nm (Fig. 5c).

Fig. 6. Tautomeric structures of adenosine

D. ^{6}N-methyladenosine, (6 MA)

The differences between the spectral positions for 6 DMA and ADO could be due to methyl shifts, and to investigate this possibility we have examined 6 MA. Despite the fact that it is not a fixed tautomer its behavior is directly analogous to 6 DMA, showing one emission spectrum each in the early and late time-window, at $\lambda_{max} \approx 331$ nm and $\lambda_{max} \approx 366$ nm respectively. Corresponding excitation maxima are 267 nm and 293 nm, the former correlating with the absorption maximum at 265 nm. The decay kinetics are two component with 30% of a 4.1 ns decay at 350–400 nm when excited at 270 nm. All these parameters are intermediate between 6 DMA and ADO, consistent with 6 MA being in the amino form and the spectra showing usual methyl shifts.

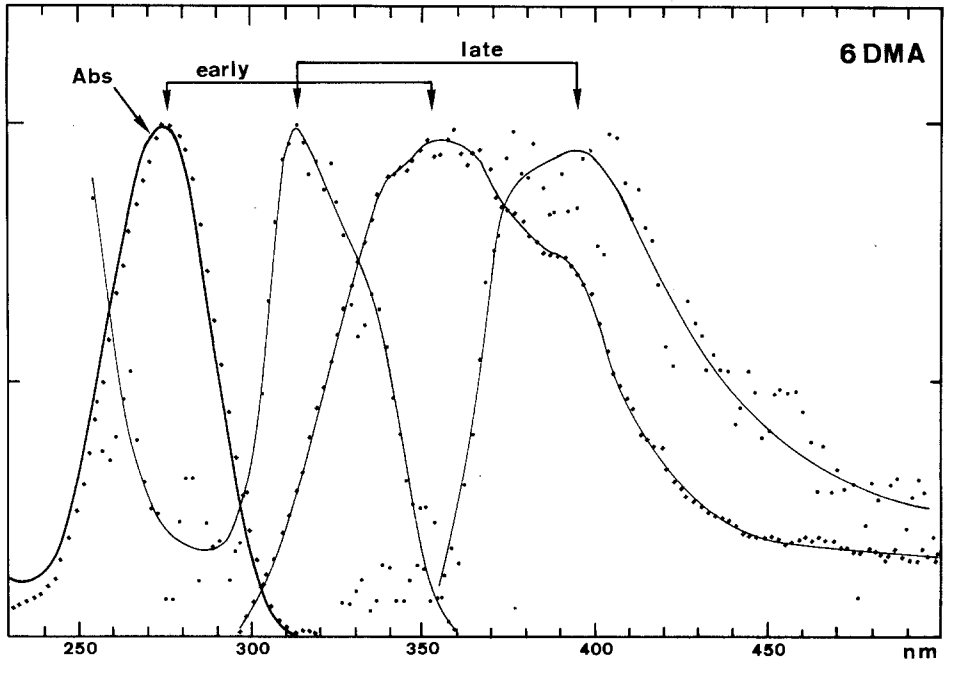


Fig. 7. Time-delayed emission and excitation spectra for 6 DMA. *Black square*: Early time-window emission (λ_{ex} = 260 nm), excitation monitored at λ_{ex} = 350 nm. *Black dot*: Late time-window emission (λ_{ex} = 315 nm), excitation monitored at λ_{em} = 400 nm. Corresponding emission and excitation spectra are indicated by *double-headed arrows*. Absorption spectrum is *continuous line*

Table 1. Stokes' shift from time-resolved spectra. Position of the maxima of emission and excitation bands. Wavelengths in nm. Wavenumbers values in cm^{-1} have been taken from replotted corrected spectra in wavenumbers

	Absorption		Early exc.		Early em.		Early Stoke	Late exc.		Late em.		Late Stoke
	nm	cm^{-1}	nm	cm^{-1}	nm	cm^{-1}	cm^{-1}	nm	cm^{-1}	nm	cm^{-1}	cm^{-1}
ADO	258	38,700	257	38,900	320	31,200	7,700	269	37,200	390	25,600	11,600
								276	36,200	360	27,800	8,400
6 DMA	275	36,400	277	36,000	350	27,900	8,100	313	31,900	395	24,300	7,600
ADO H^+	256	39,000						269	37,100	391	25,300	11,800

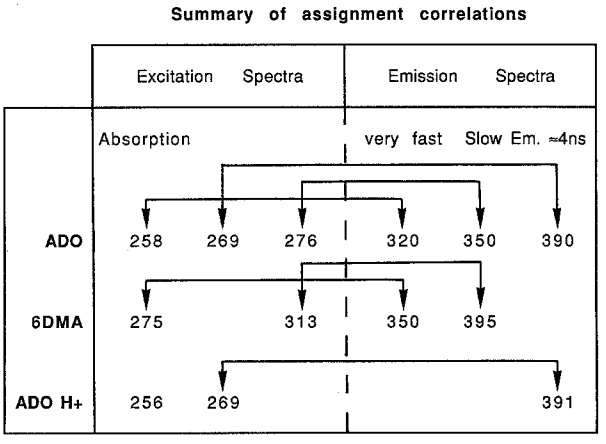


Fig. 8. Summary. Assignment of transitions in ADO, 6DMA, ADO H^+. *Double arrows* refer to correlated excitation/emission spectra. Wavelengths in nm

E. 1-methyladenosine, (1 MA)

Another molecule with a fixed tautomeric structure in the unprotonated state is 1 MA, which we anticipated could be a model for a ^{1}H-imino structure. However there are several problems with this molecule and with results reported to date. First, 1 MA has a pKa ≈ 7, so that in neutral solution it exists $\approx 50\%$ in the protonated form (of uncertain tautomeric structure). Second, the uncharged form is thermally unstable, undergoing the Dimroth re-arrangement to 6 MA (Lister 1971). These features may be responsible for discrepancies in reported excited state behavior.

Thus Börresen (1967) reported a broad emission at 393 nm (corrected) from 1 MA in acid solution, and an excitation spectrum at 270 nm compared with an absorption maximum at 257 nm. This emission, with $\phi_f = 1.4 \times 10^{-3}$ is stronger than protonated adenosine ($\phi_f = 8 \times 10^{-4}$). More recently Knighton et al. (1982) reported a much weaker emission ($\phi_f = 2 \times 10^{-5}$), peaking at 320 nm, and with coincident excitation and absorption spectra peaking at 258 nm. Surprisingly, this emission is weakly polarized ($r = 0.06 \pm 0.03$) compared with 1-methyladenine which has the same quantum yield and emission maximum and is quite polarized ($r = 0.21 \pm 0.04$). Dreyfus et al. (1977) published an absorption spectrum peaking ≈ 260 nm with a tail extending up to 330–350 nm, depending on the solvent. In these circumstances we have limited ourselves to searching for evidence for a nanosecond emission from 1 MA at pH ≈ 10 by measurement of decay profile at 390 nm. The result superimposes on the exciting flash profile and hence the decay must be very fast. We do not find any slow decay time.

F. Correlations of species by Stoke's shift and lifetimes

All the preceding work has provided evidence for multiple excitation and emission spectra which are correlated with each other on a one-to-one basis. These excitation/emission pairs in different molecules may be related by their Stoke's shifts and lifetimes. Pertinent results are in Table 1.

For ADO, the fast early-time species shows a shift of 7,700 cm^{-1}. This is close to the same early-time window species from 6 DMA (8,100 cm^{-1}), despite the 2,300 cm^{-1} red-shift in absorption due to methylation. For the nanosecond late time-window species the shifts for ADO ('360') and 6 DMA are 8,400 cm^{-1} and 7,600 cm^{-1}. The magnitude of these values contrast with the late-window species for ADO H^+ (11,800 cm^{-1}) and the other late window species ('390') for ADO (11,600 cm^{-1}). In these latter cases the maxima of excitation and emission almost coincide. Consequently it appears that two of the pairs of ADO (257/320 and 276/360) correlate with 6 DMA (277/350 and 313/390), while the other pair of ADO (269/390) correlates with ADO H^+ (269/391), as diagrammed in Fig. 8.

Discussion

Reviewing the entire available data on neutral adenosine allows us to state the problems we face in understanding this system as follows:

1) From time-delayed emission spectra there are three emitting states ('320', '350', '390'). The '320' is a fast decaying state with lifetime < 100 ps. Kinetic studies of the '350' and '390' states show only one lifetime around 4 ns, so that either they have the same lifetime,

or they cannot be resolved under our condition of low counts (low S/N ratio).

2) From time-delayed excitation spectra there are three absorbing species present, '258', '269' and '276', one of which ('258') coincides with the absorption spectrum, the '269' being correlated with the '390' emission and the '276' with the '350'.

For 6 DMA, a fixed tautomer system, we observe two states from time-delayed emission spectra, together with two corresponding absorbing states from excitation spectra.

The problem then is to identify the absorbing species in each case, to relate them to the emitting states, and to relate 6 DMA to ADO.

A. Origins of multicomponent spectra

Several different processes which may account for the difference between absorption and excitation spectra have been considered.

a) Monomers ↔ aggregate equilibrium. From work we have done earlier on ApA and Poly A (Ballini et al. 1982, 1983; Morgan and Daniels 1980), we know that their total emission spectra are quite different from adenosine and they contain components usually considered excimeric in nature, which are very similar to the longer lived emissions we are reporting here. The possibility that we are observing excimeric-type emissions from aggregates of adenosine has to be carefully evaluated. To make a correlation with excitation spectra requires that the stacked aggregates have an absorption spectrum different from monomeric adenosine.

Evidence against this proposition is as follows (*i*) numerous experiments have been carried out attempting to remove the long-wavelength long-lifetime emission. These have included measuring the total emission spectrum over the temperature range 4°–90 °C; no change in shape was observed. (*ii*) The spectra are usually observed at $1\times10^{-4}\,M$ concentration and are unchanged at $2\times10^{-5}\,M$; this implies that we are not observing a concentration-dependent aggregation. (*iii*) To avoid the possibility that we may be observing emission from suspended microcrystals, solutions have been prepared by first dissolving in acid and then bringing to neutral (buffered) pH. No change in behavior was observed. (*iv*) The concentration at which we observe these effects ($1\times10^{-4}\,M$) is incompatible with the magnitude of the known aggregation (association) effect as observed by non-spectroscopic methods. Considering for simplicity only the first stage of an association representable by $M+M\overset{K_a}{\leftrightarrow}M_2$, then $[M_2]\cong K_a[M_0]^2$. K_a is known to be ≈4 (Ts'o 1974), thus giving an associated concentration $\approx4\times10^{-8}\,M$. The application of this value to the present work requires that either the absorption coefficient of the aggregate or the fluorescence quantum efficiency be very high. This can be seen as follows. It is apparent from our measurements that the integrated intensity of fluorescence from the aggregate is, within an order of magnitude, comparable to that from the monomers and we can approximate this relation by

$$\varepsilon_1\,\phi_1\,[M_2]\cong\varepsilon_m\,\phi_m\,[M],$$

where 1 signifies the long wavelength, long decayline emission. Known values (Vigny 1973) of $\phi_m(\approx5\times10^{-5})$ and $\varepsilon_m(=15{,}500)$, $[M]=1\times10^{-4}$ and $[M_2]=4\times10^{-8}$ lead to $\varepsilon_1\,\phi_1\cong2\times10^3$. Such a value is incompatible with reasonable expectation for either ε_1 or ϕ_1. If $\varepsilon_1\approx10^4$ (order of magnitude of monomer) then $\phi_1\approx0.2$ which is four orders of magnitude greater than monomer emission; even low temperature measurements give no indication of such a strong emission. A maximum value for ϕ_1 of 1.0 gives a lower limit to ε_1 of 2000. On the other hand, any value of $\phi_1\leqq10^{-1}$ gives unreasonably high values for ε_1 ($\geqq20{,}000$). For example if $\phi_1\approx10^{-4}$ (i.e. somewhat greater than the monomers and compatible with previous steady state excitation) then $\varepsilon_1\approx10^7$. Thus the product $\varepsilon_1\,\phi_1\cong2\times10^3$, which is based on $K_a=4.0\,M^{-1}$, is incompatible with any reasonable values of either ε_1 or ϕ_1.

(*v*) In an effort to observe the concentration-dependent effects which this model requires, the absorption spectrum of adenosine has been measured from a concentration of $2\times10^{-6}\,M$ in a 100 mm pathlength cell, to a concentration of $6\times10^{-3}\,M$ in a 0.1 mm pathlength cell calibrated by He–Ne laser interference fringes. No change in absorption spectrum could be observed; this is of course consistent with an association K_a constant of 4.0. We conclude that the aggregation/association model cannot account for our observation.

b) Solvated ground-state ↔ desolvated ground-state. Recently Frechet et al. (1979) have demonstrated the dependence of the absorption spectra of nucleotides on temperature and have interpreted it in terms of a progressive desolvation with increasing temperature. The difference spectrum recorded by Frechet et al. for AMP is clearly different to our excitation spectra, and this possibility is discarded.

c) Excited-state protonation reaction of adenosine. The shape of one of the longer-lived emissions which we observe is similar to the fluorescence from adenosine in acid solution and this raises the possibility that the excited state reaction

$$\mathrm{Ado}^* + \mathrm{H_2O}\overset{k}{\rightarrow}(\mathrm{Ado\,H^+})^* + \mathrm{OH^-}$$

might be occurring, despite lack of evidence for this at 77 K (Longworth et al. 1966). Such an excited state

process cannot be considered as a completely satisfactory explanation of our room temperature observations for two reasons. First we find no evidence of consecutive reaction kinetics, which this process requires. Secondly the excited state behavior of protonated adenosine is in itself complex and not completely understood. The striking feature is that the difference between the absorption and excitation spectra of adenosine in neutral solution is maintained in acidic solution and so the fundamental problem is to understand the excitation spectra. Börresen (1967) directed attention to interpreting this behavior in terms of tautomers of adenosine and we follow this lead.

d) Differential fluorescence of adenosine tautomers. A conceptual framework for understanding our results would be provided if adenosine exists in three tautomeric forms, which have distinct fluorescence spectra. Tautomerisation in adenosine is due to the two 'mobile' H atoms of the extra cyclic amino group (Fig. 6), and to eliminate the problem due to tautomerism is the reason for investigating 6 DMA. If the phenomena of multiple excitation/emission spectra are due to the existence of tautomers, then 6 DMA should show just one emission spectrum and an excitation spectrum superimposing on the absorption spectrum. This is not the case. 6 DMA shows two emission and excitation spectra which correlate with two of the adenosine excitation/emission pairs. Consequently tautomerism cannot be the source of the multiple spectra of adenosine.

B. Dual excitation and emissions from 6 DMA

It appears that the behavior of ADO can be understood as a combination of the behavior of 6 DMA and of ADO H^+. Tautomerisation as the origin of the dual excitation/emission of 6 DMA can be ruled out a priori. Protonation of 6 DMA has no effect on its emission spectrum ($\lambda_{max} \approx 358$ nm) or quantum yield (which is as low as adenosine in neutral solution) and the excitation and absorption spectra essentially coincide (Knighton et al. 1982). Consequently protonation of 6 DMA must also be ruled out. In view of this we suggest that the two excitation spectra originate in two rotational conformers of the exocyclic $-N(CH_3)_2$ group, which may be coplanar with or orthogonal to the plane of the heterocyclic ring. Such a situation has been shown to be responsible for the well-known dual fluorescence of *p*-(dimethylamino)benzonitrile in which excitation gives rise to a twisted internal charge transfer state (Grabowski et al. 1979; Huppert et al. 1982; Wang et al. 1981) with large excited state dipole, with nanosecond lifetime and strong solvation. Such an effect can account for the anomalously long lifetime which we observe at room temperature for neutral adenosine and pronatal adenosine.

To be effective, this proposal concerning rotational conformers implies a considerable role of the amino group in the electronic transitions which is supported by the strong effects of amino group methylation or N3 methylation on the absorption spectra. Similar effects are known in the spectroscopy of anilines (Murrell 1963) and have been discussed by Kasha and Rawls (1968) in term of partial charge transfer to the ring, described as $a_\pi^* \leftarrow 1$.

C. ADO

If the $-NH_2$ group of ADO has the same conformational behavior as the $-N(CH_3)_2$ group of 6 DMA, then two of the three excitation/emission pairs can be accounted for. This seems to be the case. However there remains another excitation/emission pair (269/390) to be accounted for. This pair has almost identical characteristics with the excitation/emission pair of protonated adenosine, which we now consider.

D. Processes in acidic solution

Although the investigation of protonated adenosine was not the intent of this work, any satisfactory account must be consistent with the behavior of both neutral and acidic solution. There has been much discussion over the years on 'the' site of protonation of adenine and adenosine; these are not necessarily the same, nor in fact has it been shown that only one unique site is protonated in either molecule. A priori even the investigation of di-substituted adenosines would seem to be very difficult to resolve. For example 6 DMA structure has four potential protonation sites (^1N, ^3N, ^7N, ^6N). However working empirically from the fluorescence results assignments can be suggested to account for the behavior of both neutral and acid solutions. In overview the outstanding features about the fluorescence of adenosine in acid solution are (*i*) the positions of, and shift between the excitation and absorption spectra are unchanged from the 269/390 excitation/emission couple in neutral solution (*ii*) the emission is almost entirely long-wavelength with a much bigger Stoke's shift ($\approx 11{,}800\ cm^{-1}$) than for the other excitation/emission couples of neutral solution ($\approx 8{,}000\ cm^{-1}$), and (*iii*) has an overall efficiency an order of magnitude greater, but still only $\approx 8 \times 10^{-4}$. Items (*i*) and (*iii*) tell us that the behavior of the 269/390 excitation/emission of neutral adenosine is unchanged in nature and is simply enhanced on protonation. As this effect does not exist in acidic 6 DMA (Knighton et al. 1982), it cannot be due to any protonated 6-dimethylamino tautomer. Our decay-time measurement on the fixed ^1H-imino tautomer, 1MA in an alkaline buffer at pH ≈ 10 gives no slow decay time, which signifies that this 1-imino form cannot account

for the 269/390 excitation/emission process. This is also supported by the fact that the absorption maximum is at 257 nm. On the other hand, the ^{3}H-imino tautomer could possibly be the basis for an explanation, based on the 274 nm absorption maximum of 3-methyladenine at pH 1.5 (Knighton et al. 1982). Against this possibility is the reported weak emission at 320 nm which must be fast because of its high anisotropy (Knighton et al. 1982). Unambiguous conclusions on the role of the uncharged ^{3}H-imino tautomer must await the synthesis of 3-methyladenosine.

To keep the same tautomeric structures after protonation is only possible if protonation takes place at N 7; this is in agreement with the experimental conclusions of Knighton et al. (1982). The role of N7 is supported by the strong and completely depolarized fluorescence very similar to protonated adenosine which has been reported to occur from 7-ethyladenosine in neutral solution (Knighton et al. 1982). This substitution is likely to favor the out of plane rotamer of the amino group (Smagowicz et al. 1974; Cazeau-Dubroca et al. 1983). We suggest that the 269/390 may originate from a perpendicular $-NH_2$ conformer, due to interaction in the ground state with the solvent, this interaction being enhanced when protonation occurs (probably at N7).

The differences in the behavior of 6DMA and ADO may be traced to the non-polar nature of the methyl groups of 6DMA which cannot be involved in hydrogen-bonded solvent interactions. In contrast to this, the amino group of ADO is strongly involved in H-bonding with the solvent; its conformational behavior can thus be influenced by solvation at N7 and changes in that solvation due to protonation. The strong role of protic solvent in stabilizing twisted internal charge transfer transitions to which our model is closely analogous is well known (Cazeau-Dubroca et al. 1983; Wang and Eisenthal 1982; Hicks et al. 1985; Anthon and Clark 1987).

E. Nature of emission spectra

Excitation of neutral adenosine gives rise to three independent emissions. The '320' species, from its very short lifetime, comparison with low temperature spectra, excitation maximum at 258 nm and approximate mirror symmetry with the absorption spectrum can be assigned as the normal adenosine fluorescence with almost no solvent rearrangement before emission. This lack of rearrangement is a consequence of either its very short lifetime or lack of change of dipole moment on excitation or both. We suggest this emission comes from the co-planar conformation of the amino group.

The '320' and '350' species having a similar Stoke's shift $\approx 8{,}000\ cm^{-1}$ and having corresponding excitation/emission couples in 6DMA, are assigned as rotamers and we suggest the '350' species corresponds to the orthogonal conformation of the amino group relative to the purine plane. As such the dipole moment change on excitation and the Stoke's shift will be similar to the '320' species, but due to the orthogonal arrangement of the orbitals the radiative rate constant can be much smaller and this may account for the nanosecond lifetime which is observed.

The nature of the '390' emission is interesting. Although it has a nanosecond lifetime very close to that of the '350' species, it has a much larger Stoke's shift ($\approx 11{,}600\ cm^{-1}$), a different excitation spectrum, and is virtually identical to the strongly solvent-shifted room temperature emission from acidic solution. This Stoke's shift involves a solvation change in the excited state as manifest by an energy gap $\approx 3{,}000\ cm^{-1}$ between the onsets of excitation and emission curves of ADO H^+. These features suggest the existence of the orthogonal conformer in two states having different solvation, enhanced by protonation at N7, although the existence of some 3-imino tautomer cannot be excluded.

The essential conclusions concerning the excitation and emission spectra of ADO, 6DMA, and ADO H^+ are summarized in Fig. 8.

Acknowledgements. We thank Dr. Jean-Claude Brochon who is in charge of the UV fluorescence facilities at LURE, for the accessibility and the reliability of the experimental set-up.

This work has been supported in part by PHS grant GM 30474.

We thank Ding-guo Ho for computational assistance, and O. Chalvet for discussions concerning the theoretical interpretation of our results in relation to MO computation.

References

Anthon DW, Clark JH (1987) Picosecond excited-state solvation dynamics of 9,9'-bianthryl in alcohol solutions. J Phys Chem 91:3530–3536

Ballini J-P, Daniels M, Vigny P (1982) Wavelength-resolved lifetime measurements of emissions from DNA components and Poly rA at room temperature excited with synchrotron radiation. J Lumin 27:389–400

Ballini J-P, Daniels M, Vigny P (1983) Synchrotron excitation of DNA fluorescence decay time, evidence for excimer emission at room temperature. Biophys Chem 18:61–65

Blumberg WE, Eisinger J, Navon G (1968) The lifetimes of excited states of some biological molecules. Biophys J 8:A–106

Börresen HC (1967) Fluorescence and tautomerism of protonated and methylated adenine derivatives. Acta Chim Scand 21:2463–2473

Cazeau-Dubroca C, Peirigua A, Ait Lyazidi S, Nouchi G (1983) Twisted internal charge transfer fluorescence of para-N,N-dimethylaminobenzonitrile in rigid matrix at room temperature. Chem Phys Lett 98:511–514

Dreyfus M, Dodin G, Bensaude O, Dubois JE (1977) Tautomerism of purines. 2 Amino-imino tautomerism in 1-alkyladenines. J Am Chem Soc 99:7027–7037

Frechet D, Ehrlich R, Remy P (1979) Thermal perturbation differential spectra of ribonucleic acids. I. Hydration effects. Nucleic Acids Res 7:1965–1979

Frye SL, Jaeju Ko, Halpern AM (1984) Improved analysis of time-resolved single photon counting fluorescence data of excimer/exciplex systems using a dual analysis iterated reconvolution program on a dedicated laboratory microcomputer. Photochem Photobiol 40:555–561

Grabowski ZR, Rotkiewicz K, Siemiaczuk A, Cowley DJ, Baumann W (1979) Twisted intramolecular charge transfer states (TICT). A new class of excited states with a full charge separation. Nouv J Chim 3:443–454

Guyon PM, Depautex C, Morel G (1976) Design of a synchrotron radiation facility for ORSAY's ACO storage ring: LURE. Rev Sci Instrum 47:1347–1356

Hicks J, Vandersall M, Babarogic Z, Eisenthal KB (1985) The dynamics of barrier crossings in solution: the effect of a solvent polarity-dependent barrier. Chem Phys Lett 116: 18–23

Huppert D, Rand SD, Rentzepis PM, Barbara PF, Struve WS, Grabowski ZR (1982) Picosecond kinetics of p-dimethylaminobenzonitrile. J Chem Phys 75:5714–5719

Kasha M, Rawls HR (1968) Correlation of orbital classification of molecular electronic transitions with transition mechanism: the aromatic amines. Photochem Photobiol 7: 561–569

Knighton WB, Giskaas GO, Callis PR (1982) Fluorescence from adenine cations. J Phys Chem 86:49–55

Knutson JR, Beecham JM, Brand L (1983) Simultaneous analysis of multiple fluorescence decay curves: a global approach. Chem Phys Lett 102:501–507

Lister JH (1971) The chemistry of heterocyclic compounds. In: Brown DJ (ed) The purines, vol. 24, part 2. Wiley Interscience, New York, pp 313–316

Longworth JW, Rahn RO, Schulman RGJ (1966) Luminescence of pyrimidines, purines, nucleosides, and nucleotides at 77 K. The effect of ionization and tautomerization. J Chem Phys 45:2930–2939

Morgan JP, Daniels M (1980) Excited states of DNA and its components at room temperature. III Spectra, polarisation and quantum yields of emissions from ApA and poly rA. Photochem Photobiol 31:101–113

Murrell JN (1963) Steric effects. In: Murrell JN (ed) The theory of electronic spectra of organic molecules. Methuen, London. John Wiley & Sons, New York, pp 238–246

Smagowicz J, Berens K, Wierzchowski KL (1974) Unique properties of the lowest excited singlet state of 4-dialkylaminopyrimidines. In: Birks JB (ed) Excited states of biological molecules. John Wiley, New York London, pp 24–27

Ts'o POP (1974) Bases, nucleosides, and nucleotides. In: Ts'o POP (ed) Basic principles in nucleic acid chemistry. Academic Press, New York, pp 453–584

Vigny P (1973) Fluorescence de l'acide polyadenylique a temperature ambiante. CR Acad Sci Paris D277:1941–1944

Vigny P, Duquesne M (1974) A spectrophotofluorometer for measuring very weak fluorescences from biological molecules. Photochem Photobiol 20:15–25

Wang Y, McAuliffe M, Novak F, Eisenthal KB (1981) Picosecond dynamics of twisted internal charge-transfer phenomena. J Phys Chem 85:3736–3739

Wang Y, Eisenthal KB (1982) Picosecond dynamics of twisted internal charge-transfer phenomena. The role of the solvent. J Chem Phys 77:6076–6082