Fluorescence of Thymine Tautomers at Room Temperature in Aqueous Solutions

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Fluorescence and absorption spectra of 10^{-4} M thymine in aqueous solutions at different pH values are reported at room temperature. The shape of the fluorescence spectrum is found to change when the excitation wavelength is varied. The absorption spectra for the aqueous pH 4−8 thymine solutions in the range 240−300 nm are believed to include the diketo tautomer T1 and the enol−keto tautomer T2. Steady-state fluorescence spectra and ab initio calculations support the existence of these neutral species in their ground state. The lifetimes of the excited states of the T1 and T2 tautomers are 0.72 and 3.87 ns, respectively. Aqueous thymine solutions with pH values below pH 3 contain a protonated enol−keto form of T2 in addition to the major diketo-tautomer T1 in its neutral form. The lifetime of the protonated enol−keto form is 4.2 ns. On the other hand, thymine solutions with pH values above pH 8 contain mixtures of the neutral forms of T1 and T2 as well as their monoanioic forms. However, aqueous thymine solutions at pH values above pH 11 contain a 1:1 equilibrium mixture of the monoanionic forms 1-HT^- and 3-HT^-. The lifetimes for these monoanionic forms are expected to be shorter than 0.05 ns.

1. Introduction

Thymine, 5-methyluracil, occurs naturally in DNA. The essential biological importance of thymine motivated a number of experimental[1−12,19] and theoretical[7,13−20] spectroscopic investigations.

Many of the structural and electronic studies on thymine gave controversial results: The first room-temperature excitation spectrum of thymine reported by Hauswirth and Daniels[10] exhibited a significant deviation from the corresponding absorption spectrum. The results were explained in terms of (a) the presence of two overlapping $n\pi^*$ and $\pi\pi^*$, where only one of them fluoresces, (b) emission from a minor tautomer (2,4-dienol), or (c) kinetics of competing deactivation processes. Subsequently, Vigny and Duquesne[11] found that the excitation spectrum of thymine matches its absorption closely. Moreover, Callis et al.[9] pointed out that the mere reason for the observed[12] depolarization in the fluorescence of thymine in aqueous solutions as a function of the excitation wavelength between 280 and 290 nm is due to contributions from the fluorescent thymine anion, rather than a hidden $n\pi^*$ transition. Recently our study[21] on the fluorescence of 5-chlorouracil in aqueous solutions at room temperature using picosecond laser pulses pointed to the presence of at least an enol−keto tautomer alongside the predominant diketo tautomer.

As continuation of our studies on the spectroscopic properties of important biological molecules,[21−24] the fluorescence spectra of thymine in aqueous solutions at different pH values at room temperature are reported in this paper. Results from ab initio calculations, lifetime analysis, and time-resolved spectra are also discussed and related to the tautomeric contributions and acid−base equilibrium in the ground state.

2. Experimental Section

2.1. Materials. Thymine was purchased from Fluka AG and was crystallized from triply deionized water followed by sublimation under vacuum. To judge the purity of the used thymine sample, the fluorescence spectrum of its aqueous solution was compared with the fluorescence spectrum reported by Callis[25] and was found to be almost identical to it. The aqueous solutions of thymine were prepared using triply distilled water, standard buffer solutions (pH 1−10) from Fluka, or solutions with pH 12 and pH 13 that are prepared from sodium hydroxide and calcium chloride solutions. To ensure solvent purity, samples of the pure solvent were checked for any fluorescence due to possible impurities. No fluorescence from the solvents was observed, which confirmed their purity.

2.2. Apparatus and Procedure. Absorption spectra were recorded by a Lambda 5 UV/vis spectrophotometer. Fluorescence spectra were recorded with an SPF-500 spectrofluorometer from SLM Instruments Inc. and corrected for lamp intensity and photomultiplier sensitivity. Lifetimes and time-resolved spectra were measured using the Picosecond Applied Photophysics photon counting system described elsewhere.[26] Rhodamine 6G was used for the picosecond cavity-dumped synchronously pumped dye laser system. Experiments were done at room temperature around 23.5 °C.

2.3. Molecular Parameters and Computational Properties. With the availability of fast personal computers (PC) and efficient graphics-based programs, it is now possible to carry out rapid ab initio calculations on molecules with 12 or more atoms. In this study, PC-SPARTAN[27] for Intel 486, Pentium, and Pentium Pro processors running under Windows 95/NT have been used to supply molecular structures and their relative stability.

Since most of the Hartree−Fock (HF) and Moller−Plesset (MP) single-point calculations on uracil tautomers[28,29] were carried out on a geometry optimized at the LCAO-MO-SCF Hartree−Fock level with the Pople split-valence shell 6-31G* polarization basis set,[30] we performed our calculations to investigate thymine tautomerism using the same basis set. Thymine has six expected tautomers (Scheme 1).

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SCHEME 1

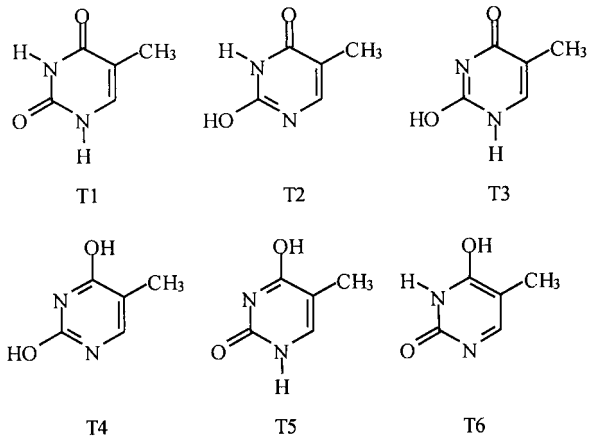


TABLE 1: Structural Parameters (Bond Length in Å and Bond Angle in Degrees) for Thymine Molecule

parameters	HF/6-31G*	exptl[a]	parameters	HF/6-31G*	exptl[a]
$r(N_1C_2)$	1.368	1.335	$r(C_4O_8)$	1.195	1.231
$r(C_2N_3)$	1.371	1.361	$r(C_5C_9)$	1.503	1.503
$r(N_3C_4)$	1.387	1.391	$r(N_1H)$	0.995	0.790
$r(C_4C_5)$	1.471	1.447	$r(N_3H)$	0.998	0.810
$r(C_5C_6)$	1.330	1.349	$r(C_6H)$	1.073	0.860
$r(C_6N_1)$	1.378	1.382	$r(C_9H)$	1.084	0.970
$r(C_2O_7)$	1.195	1.234			
$\angle(N_1C_2N_3)$	113.3	115.2	$\angle(N_3C_4O_8)$	120.5	118.3
$\angle(C_2N_3C_4)$	127.7	126.3	$\angle(O_8C_4C_5)$	124.6	126.1
$\angle(N_3C_4C_5)$	114.9	115.6	$\angle(C_4C_5C_9)$	117.9	119.0
$\angle(C_4C_5C_6)$	117.6	118.2	$\angle(C_9C_5C_6)$	124.5	122.8
$\angle(C_5C_6N_1)$	123.0	121.8	$\angle(C_6N_1H)$	120.9	125.0
$\angle(C_6N_1C_2)$	123.5	122.8	$\angle(C_2N_3H)$	115.8	108.0
$\angle(N_1C_2O_7)$	123.2	122.7	$\angle(C_5C_6H)$	122.3	127.0
$\angle(O_7C_2N_3)$	123.5	122.1			

[a] X-ray data for thymine monohydrate crystal ref 31.

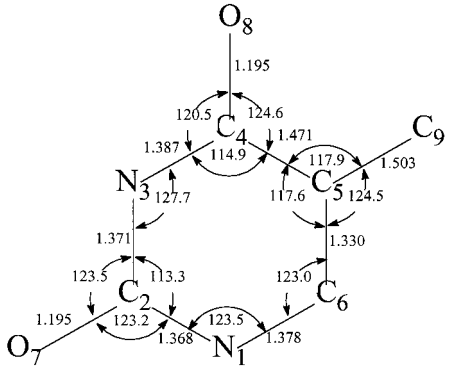


Figure 1. 6-31G* optimized structural parameters (bond length in Å and bond angle in degree) for the T1 tautomer of the thymine molecule.

The 6-31G* optimized structural parameters, namely, intermolecular bond lengths and angles, corresponding to the most stable diketo-tautomer form (T1, Scheme 1) are listed in Table 1 and shown in Figure 1 because it is the only one for which X-ray crystallographic results[31] are available. Although intermolecular hydrogen bonds produce an appreciable distortion in the molecular geometry,[31,32] bond distances and angles obtained from X-ray experiments may still be used as a guide.

A comparison of the experimentally observed bond distances of thymine with those obtained form the ab initio calculation is shown in Figure 2. Except for the C−O bond distances of the keto groups, the computed distances for the bonds within the pyrimidine ring of the T1 tautomer and C−C bond of the methyl group provide a geometry very similar to the experimental

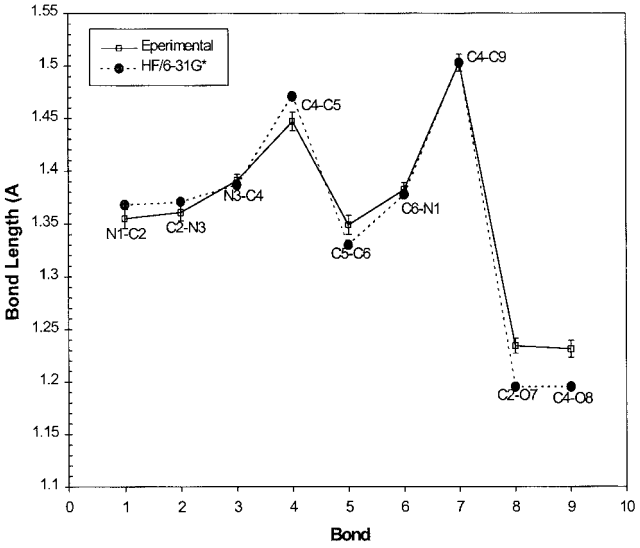


Figure 2. Comparison of the calculated (dotted line) and observed[31] (solid line) bond lengths of thymine. The error bars are twice the standard deviation of the associated bond distance.[31]

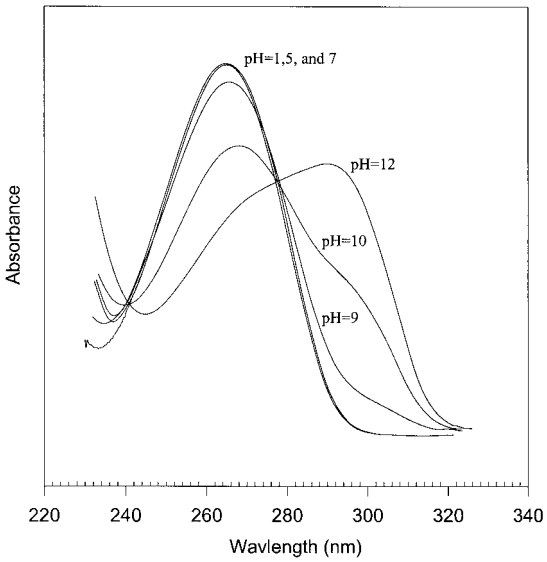


Figure 3. Absorption spectra of 10^{-4} M aqueous thymine solutions at different pH values at room temperature.

results. The observed large bond distances for the C−O bonds in the keto groups were explained[31] on the basis that the solid form of thymine is possibly a resonant state formed from seven canonical formulas. Moreover, the apparent bond contraction for bonds involving hydrogen atoms from the X-ray experiment has been explained[31] in terms of a possible displacement of the maximum electron density around the proton toward the heavier atom in the solid state.

3. Results and Discussion

3.1. Absorption and Fluorescence Spectra. Figure 3 shows the absorption spectra of thymine at room temperature in aqueous solutions of different pH values. The spectra of thymine in pH 1 through pH 8 display a featureless broad band with a maximum of 265 nm. The same maximum value was reported by Shugar and Fox.[33] Since the pK_a of thymine is 9.9,[6] the species present in these solutions are either the cationic form of thymine, the neutral form of thymine, or both of them. At pH 9 the absorption of the anionic form of thymine starts to appear at the red end of the 265 nm band. At pH 10 the intensity

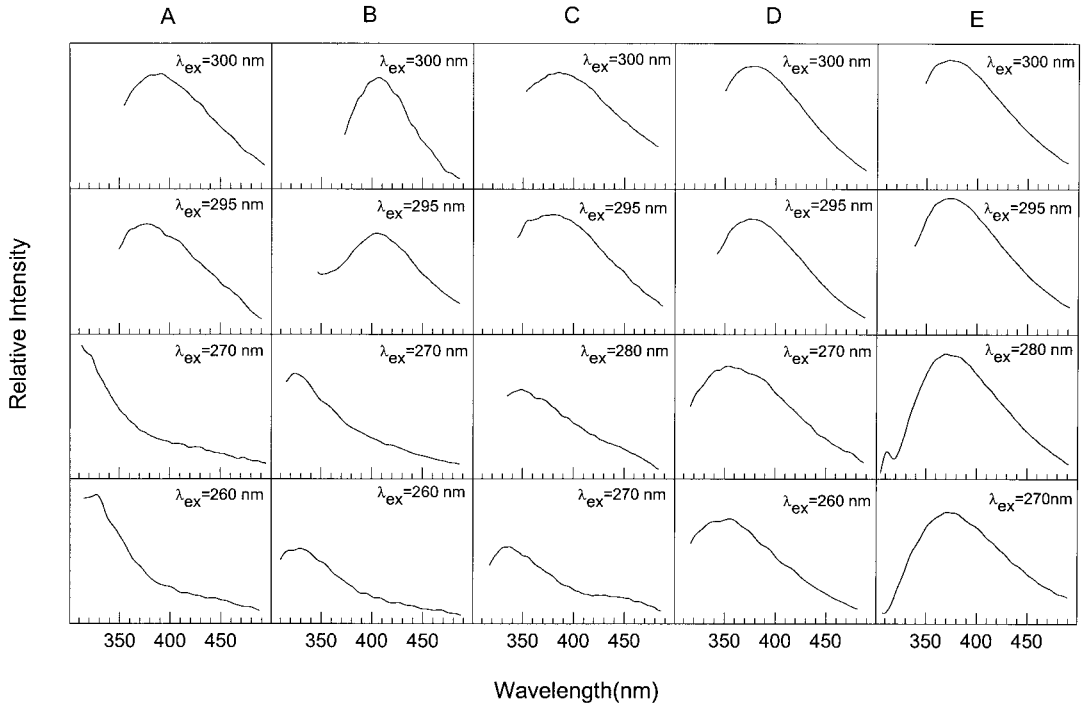
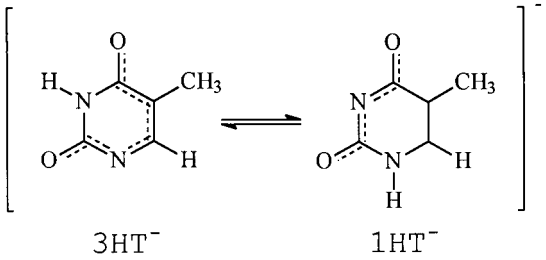


Figure 4. Corrected fluorescence spectra of 10^{-4} M aqueous thymine solutions at (A) pH 1, (B) pH 7, (C) pH 9, (D) pH 10, and (E) pH 12.

SCHEME 2



of the anionic form increases to show a strong shoulder at about 290 nm and at the same time a slight lowering in the intensity of the 265 nm band occurs. The pH 12 solution, where thymine exists almost in only the anionic form, gives a band with a maximum at about 290 nm and a sholder at about 265 nm. The pH 13 solution shows an absorption spectrum identical to that of the pH 12 solution.

Earlier UV[34] and IR[35] studies on the monoanions of thymine using appropriate fixed models, 1-methyl and 3-methyl thymine derivatives, showed that deprotonation of thymine results in a tautomeric equilibrium between its pair of monoanions (Scheme 2) with an approximately 1:1 contribution to the absorption band. These results are consistent with the integrated values, i.e., the areas under the absorption bands, from the absorption spectrum of the pH 12 aqueous solution as well as from the higher pH aqueous solutions.

Figure 4 shows the effect of variation of the excitation wavelength for the aqueous solutions of different pH values. Part B from Figure 4 presents the fluorescence spectra for the neutral solution of thymine. The excitation of this solution at wavelength values around the absorption maximum (260−270 nm) gives a broad fluorescence band with a maximum around 325 nm. When the excitation wavelength is increased to 295 nm, a much stronger broad fluorescence band appears at around 405 nm. A similar behavior has been observed for the pH 5 solution.

Various research groups[10,11,36] have reported the decrease in quantum yield as the wavelength is shortened. Polarized absorption,[37] polarized fluorescence,[9] and the reflection experiment[38] indicate that such behavior cannot be attributed to the presence of a $n\pi^*$ transition lying under $\pi\pi^*$ transition. Moreover, all theoretical calculations[9,18] interpreted the first absorption band as a single intense $\pi\pi^*$ transition. Therefore, variation in the fluorescence spectrum as a function of the excitation wavelength is most probably due to the presence of at least two different thymine tautomers.

An earlier polarized fluorescence study on an aqueous thymine solution by Callis et al.[9] reported that the only species present at pH 5 solution is undisputably neutral thymine. In addition, fluorescence excitation spectra of jet-cooled uracil, thymine, and their fixed tautomers, namely 1,3-dimethyluracil and 4(3*H*)-pyrimidone, by Tsuchiya et al.[39] revealed the coexistence of the diketo and keto−enol tautomers for uracil and thymine in their ground states.

To date, no direct computations have been performed to investigate tautomerism in thymine. However, very recently, accurate ab initio[28,29] and density function[40] calculations have been reported to investigate tautomerism in uracil. All calculations showed that the diketo tautomer (similar to T1 in Scheme 1) is the most stable tautomer. However, the relative stability of the other tautomers is very sensitive to both the basis set and the correlation energy estimation. It is therefore necessary to evaluate the relative stability of thymine tautomers.

The estimated single-point energies, relative energies to the lowest energy value of tautomer T1 (diketo form), and dipole moments for the six thymine tautomers using geometry optimized at the HF level with a 6-31G* basis set are listed in Table 2. The relative energy order of the thymine tautomers is T1 < T2 < T3 < T4 < T5 < T6. In agreement with the previous most accurate calculations on uracil, the diketo tautomer is the most stable form and T2, T4, and T6 are comparable in their absolute orders to the corresponding uracil tautomers U1, U2, U4, and U6.[29,40] On the other hand, T3 and T5 interchanged their relative stability with respect to uracil tautomers U5 and U3,[40] which can be attributed to the steric effect between the hydroxyl and methyl groups. Moreover, the differences between

TABLE 2: Calculated Total Molecular Energies, Relative Energies, and Dipole Moments for the Six Thymine Tautomers Using the HF/6-31G* Split-Valence Basis Set

tautomer	total energy (au)	rel energy (kJ/mol)	dipole moment (D)
T1	−451.509656	0	4.59
T2	−451.488493	+55.51	3.04
T3	−451.474847	+91.30	6.34
T4	−451.471651	+99.67	4.27
T5	−451.468921	+106.85	8.61
T6	−451.465607	+115.45	7.81

the estimated relative energies of thymine tautomers are large; thus the confidence in their absolute order is higher than the confidence in the order of the uracil tautomers despite the simplicity of our calculations.

Similar to 5-chlorouracil[21] and based on the ab initio calculation on the tautomerisim of thymine, the fluorescence band observed at about 405 nm for thymine in pH 5−7 solutions excited at wavelengths longer than 280 nm is probably due to T2 tautomer, which is the most stable tautomer after the T1 tautomer. Furthermore, the appearance of T2 absorption and fluorescence bands at higher wavelengths than the respective T1 bands can be related to the extended conjugation in the T1 tautomer and its smaller computed dipole moment (see Table 2). This is in agreement with the fluorescence excitation spectra of the jet-cooled thymine.[39]

According to the *ab* initio calculation, the energy difference between the diketo and keto−enol tautomers is 55.5 kJ/mol. However, using the integrated values of the fluorescence (area under the fluorescence band) and the relative quantum of the keto−enol to the diketo tautomers (10 000:1) suggested by Tsuchiya et al,[39] we find that about 2% of the keto−enol tautomer may be present at the red edge of the absorption band (at about 290 nm). This is consistent with the 6% value for the minor tautomer suggested by Hauswirth and Daniels,[10] the less than 10% value suggested by Brown et al.,[7] and also the 3% value contribution from the out-of-plane depolarized absorption suggested by Morgan and Daniels.[12]

Part E from Figure 4 shows that the variation of the excitation wavelength on the pH 12 solution where thymine exists in almost its anionic forms, namely, $1HT^-$ and $3HT^-$, does not affect the position and shape of the fluorescence band. The band maximum is observed at about 375 nm. This band probably involves the overlapping of the $1HT^-$ and $3HT^-$ fluorescence bands.

Careful analysis for the fluorescence spectra from pH 9 (part C in Figure 4) and pH 10 (part D in Figure 4) thymine solutions add support to our view that the broad fluorescence band from pH 12 thymine solution is due to overlapping from the two monoanionic forms of thymine 1-HT^- and 3-HT^-. The pH 9 thymine solution is expected to be much more sensitive because of the very low concentration observed for the monoanionic forms of thymine (see Figure 3). The excitation of this solution at 295 and 300 nm gives a fluorescence band with a maximum at about 385 nm. This band is due to the anionic form of thymine that is related to the 3-HT^- form as well as to the N-monomethylated form 3-MeT^-.[41] On the other hand, the excitation of the same solution at 280 nm gives a fluorescence band with a maximum at about 350 nm. This band is probably due to the other monoanion form 1-HT^-. When the pH of the thymine solution was increased from 9 to 10, thereby increasing the concentration of the anionic forms, two effects were observed. First, the 350 nm fluorescence band became predominant even at an excitation wavelength of 260 nm and its maximum shifted to 360 nm. Second, at excitation wavelengths of 295 and 300 nm the fluoresence band shifted from 385 to 380 nm. Therefore, one can conclude that the apparent maximum at about 375 nm from the pH 12 thymine solution is due to the overlap of 3-HT^- (fluoresces at about 380 nm) and $1HT^-$ (fluoresces at about 360 nm).

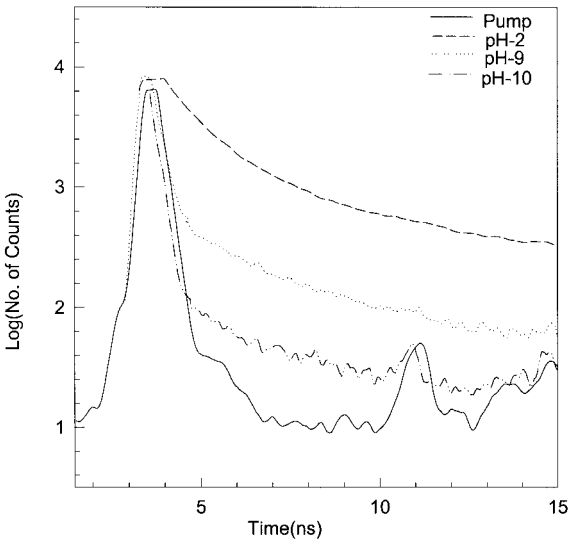


Figure 5. Fluorescence decay of pumped dye laser and 10^{-4} M aqueous thymine solutions at different pH values.

Part A of Figure 4 represents the effect of the variation of excitation wavelength on the pH 1 thymine solution. Excitation wavelengths of 260 and 270 nm give a weak fluorescence band with a maximum at about 325 nm as similar to the emission from the neutral diketo tautomer T1 in the pH 7 thymine solution. This result partly agrees with the ^{1}H-NMR, ^{13}C-NMR, and UV results by Benoit and Frechette[42] who observed that both uracil and thymine are present as neutral bases in dilute acid solution. However, the excitation of the enol−keto tautomer T2 with wavelengths of 295 and 300 nm gives a fluorescence band with a maximum at about 380 nm. This band is at a lower value relative to the one observed from the pH 7 thymine solution. It is, therefore, most probably due to the protonated form of the enol−keto tautomer T2. This is contrary to the finding of Benoit and Frechette[42] who reported that the protonated form of thymine is only encountered in highly acidic (>12 M) solutions.

Most of the above-mentioned conclusions are supported by the excited states lifetime analysis and the time-resolved spectra discussed in the following sections.

3.2. Lifetime Analysis. Figure 5 shows some of the fluorescence decays of thymine aqueous solutions at various pH values. Because of a pulse broadening of 350 ps for the detection system of the single photon counting spectrometer, it was not possible to deconvolute the lifetimes of thymine monoanions forms, as depicted in Figure 5 for pH 10 thymine solution and the other solutions at higher pH values. The pH 1 through pH 9 thymine solutions were successfully deconvoluted.

The calculated lifetimes (τ_i), normalized preexponential factor ($a_i/\Sigma a_i$), χ^2 values, and the relative quantum yields ($Q_i = a_i\tau_i/\Sigma a_i\tau_i$) for the pH 1 through pH 9 solutions are summarized in Table 3. Similar biexponential decay curves are observed for the pH 1 and pH 2 solutions with average lifetimes of about 0.72 and 4.20 ns. For the pH 5 and pH 7 solutions, different biexponential decay curves are obtained with average lifetimes of about 0.72 and 3.84 ns. The pH 9 solution fitted a three-exponential decay curve with χ^2 values comparable to those of

TABLE 3: Values for Lifetimes (τ_i), normalized Preexponential Factor ($a_i = a_i/\Sigma a_i$), χ^2 Values, and Relative Quantum Yields ($Q_i = a_i\tau_i/\Sigma a_i\tau_i$) from Deconvoluted Experimental Decay for the Normal Fluorescence of Thymine Aqueous Solutions ($\lambda_{Ex} = 295$ nm)

solvent	λ_{em} (nm)	τ_1	a_1	Q_1	τ_2	a_2	Q_2	τ_3	a_3	Q_3	τ_4	a_4	Q_4	χ^2
pH1	380				0.62 ± 0.01	0.84	0.43				4.33 ± 0.01	0.16	0.57	1.1
	420				0.70 ± 0.01	0.74	0.32				4.18 ± 0.01	0.26	0.68	1.1
pH2	380				0.82 ± 0.01	0.86	0.55				4.07 ± 0.02	0.14	0.45	1.1
	420				0.86 ± 0.06	0.73	0.35				4.47 ± 0.31	0.27	0.65	1.0
pH5	380				0.75 ± 0.01	0.69	0.30	3.91 ± 0.02	0.31	0.70				1.1
	420				0.60 ± 0.06	0.58	0.17	4.16 ± 0.15	0.42	0.83				1.1
pH7	380				0.70 ± 0.02	0.65	0.26	3.76 ± 0.05	0.35	0.74				1.1
	420				0.82 ± 0.02	0.41	0.13	3.85 ± 0.02	0.59	0.87				1.0
pH9	380	0.08 ± 0.0003	0.98	0.68	0.60 ± 0.01	0.011	0.058	3.87 ± 0.03	0.009	0.26				1.0
	420	0.03 ± 0.0015	0.99	0.50	0.69 ± 0.13	0.005	0.050	3.89 ± 0.10	0.005	0.45				1.1

the biexponential fits and having lifetimes of about 0.08, 0.6, and 3.87 ns.

The $\tau = 3.84$ ns from the pH 5 and pH 7 solutions is probably due to the same relaxed species observed from the pH 9 solution with $\tau = 3.87$ ns. This lifetime is for the enol−keto tautomer T2 and agrees very well with the previously reported values of 3.9 ns for the 5-chlorouracil keto−enol tautomer.[21] However, the $\tau = 4.2$ ns relaxation lifetime value obtained for the pH 1 and pH 2 solutions is attributed to the protonated form of the enol−keto tautomer T2.

The $\tau = 0.7$ ns is observed in all the studied solutions and is probably due to the same species. This lifetime is in agreement with earlier results,[43] which report a $\tau < 0.5$ ns for thymine and thymidine in a 1:1 mixture of ethanol and methanol. It also agrees with the value of $\tau < 0.5$ ns obtained for the fixed tautomer *N*,*N*-dimethylthymine[43] in 2-MTHF (2-methyltetrahydrofuran). This leads us to conclude that the lifetime of about 0.7 ns is for the neutral diketo tautomer T1. This lifetime is in good agreement with the previously assigned lifetime for the diketo tautomer of 5-chlorouracil.[21]

The lifetime of 0.08 ns observed in the pH 9 solution is believed to be due to the anionic forms. Comparison of the lifetimes and relative quantum yields in the pH 9 solution (τ_i and Q_i in Table 3) shows that although the contribution from the anionic forms is almost the same, the lifetime is much shorter at λ_{em} 420 nm ($\tau = 0.03$ ns) than at λ_{em} 380 nm ($\tau = 0.08$ ns). The opposite is true for all the other species with longer lifetimes. It is therefore reasonable to assume that there may be two different anionic components in the pH 9 thymine solution with two different and very short lifetimes. The one at a longer emission wavelength (the 3-HT^- anion) has a much shorter lifetime than the other anionic component (the 1HT^- anion). Although, it is difficult to measure the lifetimes of thymine monoanionic forms directly at higher pH values because of the nature of the fluorescence decay profile (see Figure 5), the results from pH 9 thymine solution and from the similar fluorophore 5-chlorouracil pH 10 solution[21] leads us to expect that their decay is biexponential. With a better time resolution detection system, the fluorescence decay lifetimes of thymine monoanionic forms, which are expected to be shorter than 50 ps, can be determined.

3.3. Time-Resolved Spectra. The time-resolved experiment is used to confirm the presence of the tautomeric forms in the studied thymine solutions and their lifetimes in the excited state. The time-resolved spectra in the pH 5 and pH 7 thymine solutions are similar to the reported[21] spectra for the pH 4 5-chlorouracil solution (see Figure 3 in ref 21). They, on average, show two major fluorescent bands, one with an emission maximum around 330 nm and the other with an emission maximum around 390 nm. For these solution systems the initially strong emission band at 330 nm weakens while the band at 390 nm becomes stronger as the time of time-resolved

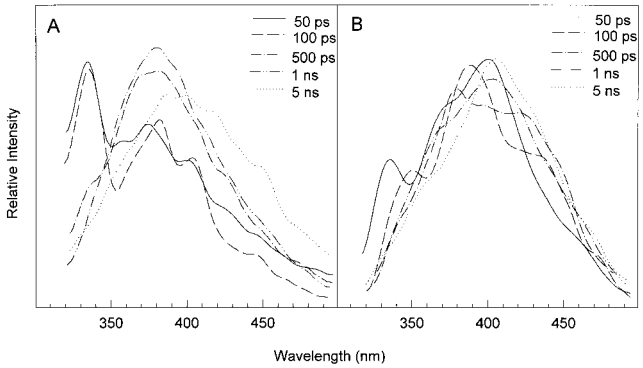


Figure 6. Time-resolved spectra of 10^{-4} M aqueous thymine solutions at (A) pH 2 and (B) pH 9.

experiment increases. Clearly, the 330 nm band is for the diketo tautomer T1 whereas the 390 nm band is for the enol−keto tautomer T2. This finding confirms the above-mentioned predictions of the steady-state fluorescence and lifetime analysis results.

Evidence from steady state and lifetime studies suggest that at a pH 2 or below the protonated form of the enol−keto tautomer predominates while at a pH 9 and above the deprotonated forms from both the diketo and enol−keto tautomers predominate. For this reason the time-resolved spectra at these pH limits are given in Figure 6. The spectra in Figure 6 are very complex due to the extensive overlap of the fluorescence from the different species. However, a close examination of these spectra reveals the following: For the pH 2 thymine solution, a strong response is observed for the diketo tautomer T1 at 330 nm (solid and dashed lines in part A of Figure 6) at 50 and 100 ps. On the other hand, at these times a stronger response was obtained from the pH 9 solution at about 390 nm (solid and dashed lines in part B of Figure 6). This confirms that the anionic forms of thymine at pH 9 have very short lifetime and compete with the diketo tautomer T1. Therefore, the short-lived 390 nm band from the pH 9 solution is related to the anionic forms of thymine tautomers, whereas the long-lived 380 nm emmission band from the pH 2 solution is related to the protonated enol−keto form of T2.

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