Molecular Recognition in Organic Solvents. The Importance of Excimer Fluorescence Spectroscopy

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The present work clearly demonstrates the usefulness of fluorescence spectroscopy as a sensitive tool for the study of molecular recognition in solution, whenever other established experimental techniques are not applicable. Excimer fluorescence spectroscopy was used, in combination with NMR, to study the hydrogenbonding interactions between a carboxylic group and a nucleic acid base. Two different organic solvents were employed: chloroform, which can participate in H-bonding, albeit weakly, and cyclohexane, which is totally devoid of this ability. The complexes formed between the reactants were determined from the quantitative analysis of the fluorescence and NMR spectra. It was found that in these solutions the predominant H-bonding interactions lead to the formation of the three dimers, acid—base, acid—acid and base—base in order of decreasing strength. The formation of trimeric units, although reported in the literature for similar systems, was not confirmed by our study.

I. Introduction

High-resolution proton magnetic resonance is the method preferably employed in studies of H-bonded complexes.¹⁻⁵ It is, however, seriously impaired whenever the reactants suffer from low solubility in the particular solvent used. From the other spectroscopic techniques, fluorescence, which is an exceptionally sensitive method applicable even to the most dilute solutions, has been used only occasionally in studies of H-bonding.⁶⁻⁸ It is evident that whenever the main shortcoming of all fluorescence methods, viz., the need for the presence of a fluorophor in the system, can be met, this technique should be successfully applied. Furthermore, appropriate probe molecules functionalized with fluorescent groups are commercially available in a great variety, in particular molecules functionalized with the pyrene moiety, which in addition to normal fluorescence exhibits excimer fluorescence as well. This is the case for instance with 1-pyrenedodecanoic acid, which is used in the present study. It is therefore very important to establish the applicability of fluorescence spectroscopy to the study of hydrogen bonding in molecular recognition. In the present work we have exemplified the usefulness of fluorescence spectroscopy by the study of molecular recognition between the nucleic acid base adenine and the carboxylic group of organic acids.

Nucleic acid bases play a very significant role in biological processes as agents of molecular recognition which occurs through hydrogen bonding between the appropriate chemical groups. Typical examples involving H-bonding include the contribution of these bases to the formation of the helix of nucleic acids by interaction with their complementary bases, as well as the recognition of nucleic acids by proteins and enzymes.^{9,10} In connection with the latter case, the interaction of butyric acid with nucleic bases has been investigated in order to simulate the corresponding nucleic base reactions with glutamic or aspartic acid.¹¹ In aqueous solutions the strong H-bonding of these bases with H_2O leads to molecular stacking;¹² in organic solvents, however, genuine molecular recognition between the solutes is greatly favored.¹³⁻¹⁶

The two solvents, chloroform and cyclohexane, employed here are very different from the point of view of H-bond formation. Thus, chloroform is known to participate, albeit weakly, in H-bonding, therefore antagonizing the solute species for the formation of H-bonds, whereas cyclohexane does not form any H-bonds and therefore it can be considered as inert for this kind of interaction. To improve the solubility of adenine in organic media, a hexadecyl chain was attached at the 9 position of adenine, producing 9-hexadecyladenine (9-HDA). Note that the 9 position is quite distant from the primary recognizable group of adenine, viz., the amino group (Figure 1), and therefore its H-bonding ability, either with itself or with complementary molecules, is expected to remain essentially intact. The organic acid employed in this investigation was dodecanoic acid functionalized at its terminal methyl group with pyrene, affording 1-pyrenedodecanoic acid (1-PDDA).

Our main objective here was 2-fold: first, to prove the importance of fluorescence spectroscopy in studies of molecular recognition when other established techniques are not applicable and, second, to study by means of fluorescence spectroscopy, supplemented by ¹H NMR, the molecular recognition between 9-HDA and 1-PDDA in organic solvents.

II. Experimental Section

9-Hexadecyladenine was prepared¹⁷ by a method analogous to the one described by Browne et al.¹⁸ 1-Pyrenedodecanoic acid was purchased from Molecular Probes and was used without any further purification, since its purity, at least as far as fluorescence is concerned, was confirmed by its absorption and emission spectra. During this work, great care was taken to avoid the presence of any water in the solvents used, because water, even in minute traces, was found to block the sites at which hydrogen-bonding interactions occur. Thus, chloroform (deuterated, Isochem 99.8 % D, protonated Carlo-Erba) was purified by passage through an alumina column followed by distillation, whereas cyclohexane (Carlo-Erba, spectroscopy grade) was stirred with anhydrous CaCl₂ and then distilled. All purified solvents were kept over 4 Å molecular sieves.

High-resolution proton NMR spectra were obtained with a Bruker AC 250 Fourier transform spectrometer operating at 250.13 MHz. For all experiments the spectrometer magnetic field was locked on an internal deuterium reference (deuterated chloroform). The NMR experiments were performed in solutions of deuterated chloroform. For the fluorescence measurements the Perkin-Elmer LS-50B spectrofluorometer, in conjunction with a constant temperature bath and circulator, was used.

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Figure 1. Structures of the main complexes formed by H-bonding when 9-HDA and 1-PDDA are dissolved in organic solvents.

Fluorescence measurements were performed in solutions of cyclohexane. The excitation of 1-PDDA was set at 370 nm in order to avoid inner filter effects due to reabsorption, as can be seen from Figure 2, which shows the absorption spectra of 9-HDA and 1-PDDA. All comparable spectra were recorded for the same absorption of the exciting light. Neither the monomer nor the excimer fluorescence of 1-PDDA was found to be quenched by 9-HDA at all concentrations studied here. The absorption spectra were recorded on a Perkin-Elmer Lambda 16 spectrophotometer.

Different solvents were used to prepare the solutions for the NMR and for the fluorescence studies because of the different solubilities of the reactants and differences in the sensitivities of the methods. Thus, for the NMR, which requires rather concentrated solutions due to its low sensitivity, chloroform was used as solvent since it dissolves easily both 1-PDDA and 9-HDA. On the contrary, the solubility of these two molecules in cyclohexane is too low to allow use of this solvent for the NMR experiments. On the other hand, because chloroform itself participates in H-bonding with 1-PDDA and 9-HDA, the



Figure 2. Absorption spectra of 9-HDA (B, $[C] = 1.00 \times 10^{-4} \text{ M}$) and 1-PDDA (A, $[C] = 3.72 \times 10^{-5} \text{ M}$) both dissolved in cyclohexane, cell = 1 cm. The arrow indicates the wavelength of excitation.

complexes formed between 1-PDDA and 9-HDA when these molecules are dissolved in chloroform are weakly bound; therefore, high concentrations of the reactants are required in order to obtain discernible differences in the fluorescence spectra. However, high concentrations of 1-PDDA will cause serious problems of reabsorption in fluorescence experiments. For this reason cyclohexane, which does not participate in any sort of H-bonding, was used as the solvent for the fluorescence measurements. In this case dilute solutions of 1-PDDA were used and inner filter effects in fluorescence spectra were negligible.

The kinetic models and equilibrium constants were obtained from the NMR and fluorescence experimental data, using the nonlinear least squares curve-fitting program Minsq (MicroMath Scientific Software), which is based on a modification of the Levenberg-Marquardt algorithm

III. Results and Discussion

9-HDA offers two binding positions with double H-bonding at the $N_1 \dots H_{syn}$ and $N_7 \dots H_{anti}$ sites and one with single H-bonding at the N_3 site (Figure 1), whereas the acid forms H-bonds through its carboxylic group. The most important complexes between these two molecules are the dimers acidacid, adenine-adenine, and acid-adenine and the trimers acid₂-adenine, as depicted in Figure 1. Formation of additional complexes is conceivable, e.g. the tetramer acid₃-adenine or oligomers involving two adenine moieties and some acid units; these, however, will not be considered here since, even if they are formed, they are expected to have very low equilibrium constants, outside the experimentally accessible range.

The reactions leading to the formation of the complexes shown in Figure 1 and the corresponding equilibrium constants are shown below, where Ac, Ac_2 , Ad, Ad_2 , Ac-Ad, etc., stand for the acid monomer, dimer, the adenine monomer, dimer, the acid adenine dimer, etc.

$$\operatorname{Ac} + \operatorname{Ac} \rightleftharpoons \operatorname{Ac}_{2}(\mathbf{I}), \quad K_{1} = [\operatorname{Ac}_{2}]/[\operatorname{Ac}]^{2}$$
(1)

$$\operatorname{Ad} + \operatorname{Ad} \rightleftharpoons \operatorname{Ad}_2(\operatorname{II}), \quad K_2 = [\operatorname{Ad}_2]/[\operatorname{Ad}]^2$$
 (2)

Ac + Ad \rightleftharpoons Ac - Ad (III, IV, V), $K_3 = [Ac - Ad]/[Ac][Ad]$ (3)

$$Ac-Ad + Ac \rightleftharpoons Ac_2 - Ad$$
 (VI, VII, VIII),
 $K_4 = [Ac_2 - Ad]/[Ac-Ad][Ac]$ (4)

It is clear that intensity changes of the excimer fluorescence upon addition of 9-HDA to a solution of 1-PDDA cannot be



Figure 3. Dependence of the chemical shifts of H_2 , H_8 , H_{syn} , and H_{anti} on the concentration of added 1-PDDA to a solution of 10^{-2} M 9-HDA in chloroform-*d*.

attributed to the formation of some specific Ac-Ad dimer or Ac₂-Ad trimer structures. Therefore, for the purposes of the present study, the equilibrium constants K_3 and K_4 stand for the apparent constants of the overall reactions leading to structures III, IV, V and VI, VII, VIII, respectively.

High-resolution proton NMR spectra support some of the structures conjectured in Figure 1. Thus, the pronounced downfield chemical shift of the single resonance of the two amino protons of 9-HDA (H_{syn} , H_{anti}) observed upon addition of 1-PDDA constitutes strong evidence that these hydrogens form H-bonds,¹¹ which could possibly correspond to some or all of the structures **III**, **IV**, **VI**, **VII**, and **VIII**. On the contrary, the H₂ and H₈ protons demonstrate upfield chemical shifts upon addition of 1-PDDA (Figure 3a). These two hydrogens are attached to carbon atoms, and therefore they are not expected to participate in H-bonding. They are also equally close to the H-bonded N₁ and N₇ atoms; therefore, their chemical shifts must



Figure 4. Continuous variation plots of the amino protons of 9-HDA: (A) experimental; (B) computed taking into account the dimerizations Ac-Ac and Ac-Ad; (C) computed taking into account only one dimer, Ac-Ad, ignoring acid self-association.

be similarly affected by the formation of the complexes III and IV. Consequently, it is reasonable to attribute the sizable upfield chemical shift of H₂ (0.15 ppm) compared to that of H₈ (only 0.02 ppm) to the formation of complex V, in which the N₃ atom, adjacent to the H₂, participates in the H-bond. Because of the fast rotation of the C₆-NH₂ bond at room temperature, the amino protons give only one signal before and after complexation. At temperatures below -40 °C, however, two distinct peaks clearly appear, the concentration dependence of which shows, by means of the observed crossing (Figure 3b), that H-bonding occurs at both sites with the site involving the H_{syn} amino proton predominating. Similar results have been reported in the literature for similar cases of H-bonded complexes.^{1b}

The self-association of 1-PDDA was also studied by NMR in carefully dried deuterated chloroform at 25 °C. The dimerization constant K_1 was calculated from the chemical shift of the OH proton, and it was found to be equal to 57 ± 15 M^{-1} . Values very close to the one reported here have been found for the dimerization of butyric acid in chloroform-*d* by other spectroscopic methods, viz., $60 \pm 20 M^{-1}$ by infrared¹⁹ and $80 \pm 15 M^{-1}$ by proton NMR.¹¹ Note that when chloroform-*d* was used without extensive removal of water, the signal of the OH proton of 1-PDDA was hardly discernible and inappropriate for the determination of K_1 .

For the estimation of the equilibrium constant K_3 of the dimer Ac-Ad we have used the Wilcox model,²⁰ modified to include the acid self-association for which the constant $K_1 = 57 \pm 15$ M^{-1} was determined in the previous experiment. For this calculation the dimerization of 9-HDA (structure II) was assumed to be negligible, as is the case for the self-association of 9-ethyladenine, a molecule very similar to 9-HDA, for which the dimerization constant has been reported to be equal to 3.1 $M^{-1,21}$ The value $K_3 = 130 \pm 15 M^{-1}$ found here is reasonably close to the corresponding value of $160 \pm 15 \text{ M}^{-1}$ for the complex between 9-ethyladenine and butyric acid found elsewhere.¹¹ When the same measurements were performed in nonextensively dried chloroform-d, the parameter fitting produced the values $K_3 = 110 \pm 5 \text{ M}^{-1}$ and $K_1 < 4 \text{ M}^{-1}$. These values indicate that small amounts of water, or perhaps some other H-bonding impurity, are detrimental for the acid self-association (structure I) but not very crucial for the formation of the acidadenine complex (structures II, III, IV).

The existence of more than one 1:1 complex in chloroform solution is further confirmed from the experimental Job plot (curve A, Figure 4), which exhibits considerable deviation from the symmetric shape expected (curve C, Figure 4) if only one equilibrium, i.e. Ac + Ad = Ac - Ad, were effective in the



Figure 5. Fluorescence spectra of (A) 10^{-4} M 1-PDDA/cyclohexane, (B) 10^{-4} M 1-PDDA/cyclohexane + 2%methanol, (C) 10^{-4} M 1-PDDA/ 70/30%(v/v) cyclohexane/chloroform, excitation at 360 nm. Spectra B and C are normalized to the same intensity of monomer emission. Inset: structure of the acid—acid dimer showing the approach of the pyrene groups.

solution. The theoretical curve of the Job plot (curve B, Figure 4) was calculated assuming two 1:1 complexes, viz., Ac-Ad and Ac-Ac, and it was found to be in good agreement with the experimental curve A in Figure 4. Such deviation of the experimental Job plot could also indicate the formation of other complexes, e.g. 2:1, etc.; this possibility, however, is eliminated from the analysis of the fluorescence data discussed later in this article.

In conclusion, analysis of the NMR data has shown that when 1-PDDA and 9-HDA are dissolved in chloroform, H-bonding leads to the formation of Ac-Ac and Ac-Ad dimers, the latter being much stronger than the former. The presence of water, even in very small amounts, breaks down the Ac-Ac dimer, while it only slightly reduces the strength of the association of the Ac-Ad complex. Actually there are two Ac-Ad dimers, the strong one involving N₁ and the H_{syn} amino proton and the weaker of the two involving N₇ and H_{anti}. There is also evidence indicating that H-bonding occurs at the N₃ position.

Before we discuss our fluorescence findings quantitatively, it would be interesting to mention first some qualitative aspects of the emission spectra of 1-PDDA. Thus, as seen in Figure 5, where the fluorescence spectra of 10^{-4} M 1-PDDA in different solvents are shown, 1-PDDA in cyclohexane (curve A in Figure 5) emits a considerable amount of excimer fluorescence. However, upon addition of only 2% methanol nearly all excimer fluorescence disappears (B, Figure 5). Similarly, 10^{-4} M 1-PDDA in a mixture of 70/30% (v/v) cyclohexane/chloroform hardly emits any excimer fluorescence (C, Figure 5). The explanation for this behavior of 1-PDDA is that acid selfassociation (structure I) which occurs in cyclohexane brings close together the two pyrene groups of the dimeric unit (inset, Figure 5), thus facilitating excimer formation. On the other hand, addition of only a few percent of methanol to the solution of 1-PDDA in cyclohexane causes nearly complete disappearance of the excimer fluorescence due to the disruption of the intermolecular H-bonds of the dimer. The same situation occurs in the cyclohexane/chloroform mixture because of the ability of chloroform to form H-bonds, thus breaking the acid selfassociation. It is therefore concluded that 1-PDDA exhibits excimer fluorescence even at low concentrations (ca. 10⁻⁴ M),

only when it forms dimers. Of course, the intensity of the excimer fluorescence is linearly dependent on the concentration of these dimers. Moreover, if 9-HDA forms H-bonds with 1-PDDA in cyclohexane (structures III, IV, V), thus breaking the dimerization of the acid, the excimer fluorescence will be expected to decrease as the concentration of added 9-HDA is increased. On the other hand, should the trimeric structures VI-VIII be present in the solution, they would increase excimer formation and cause a more complicated dependence of the overall intensity of the excimer fluorescence on the concentration of the reactants.

The self-dimerization of 1-PDDA can be studied separately in a solution containing only this particular reactant. In this way, the association constant K_1 can be determined directly and independently of the other constants. Thus, combining eq 1 with eq 5, which expresses the unknown free monomer $[Ac]_f$

$$[Ac]_{t} = [Ac]_{f} + 2[Ac_{2}]$$
 (5)

and dimer $[Ac_2]$ concentrations in terms of the known total acid concentration $[Ac]_t$, we have deduced eq 6, which gives the concentration of the dimers $[Ac]_2$ in terms of K_1 and $[Ac]_t$.

$$[Ac_{2}] = \frac{1}{8}(4[Ac]_{t} + K_{1} - \sqrt{K_{-1}^{2} + 8[Ac]_{t}K_{-1}}) \text{ where } K_{-1} = 1/K_{1}$$
(6)

On the other hand, since all excimer fluorescence originates only from dimerized 1-PDDA, the ratio I_1/I_2 of two different excimer fluorescence intensities will be equal to the ratio $[Ac_2]_1/$ $[Ac_2]_2$ of the corresponding dimer concentrations.

$$I_1/I_2 = [Ac_2]_1/[Ac_2]_2$$
 (7)

Finally combining eqs 6 and 7, eq 8 was obtained, which expresses the unknown K_1 in terms of the measurable quantities [Ac]₁, *I*, and I^0 .

$$\frac{I}{I^{0}} = \frac{4[\mathrm{Ac}]_{\mathrm{t}} + K_{-1} - \sqrt{K_{-1}^{2} + 8[\mathrm{Ac}]_{\mathrm{t}}K_{-1}}}{4[\mathrm{Ac}]_{\mathrm{t}}^{0} + K_{-1} - \sqrt{K_{-1}^{2} + 8[\mathrm{Ac}]_{\mathrm{t}}^{0}K_{-1}}}$$
(8)

In a series of experiments, starting from some initial, and highest, total acid concentration $[Ac]_{t}^{0}$ and the corresponding highest excimer intensity I^{0} , we have measured the intensities I following successive dilutions, leading to total concentrations $[Ac]_{t}$. Fitting then eq 8 to these experimental data (Figure 6a), we have determined the best values for K_{1} at two different temperatures, viz., $K_{1}(10 \text{ °C}) = (1.10 \pm 0.12) \times 10^{4} \text{ M}^{-1}$ and K_{1} (25 °C) = (4.6 ± 0.7) × 10³ M⁻¹. Both these values are in good agreement with previously published equilibrium constants for similar cases. Thus, the self-association of butyric acid in heptane at 23 °C was found to have an equilibrium constant $K_{eq} = 5.75 \times 10^{3} \text{ M}^{-1}$,²² whereas the same equilibrium constant at 10 °C in cyclohexane was found to be equal to (1.4 ± 0.4) $\times 10^{4} \text{ M}^{-1}$,²³

The addition of 9-HDA to a solution of 1-PDDA in cyclohexane results in additional equilibrium processes, viz., the reactions described by eqs 2–4. In this case, the excimer fluorescence will originate not only from the Ac-Ac dimers but also from the Ac₂-Ad trimers, since such structures allow the mutual approach of the pyrene groups and the formation of excimers. Under these conditions the experimentally accessible ratio I/I^0 is given by eq 9.



Figure 6. (a) Dependence of the excimer fluorescence intensity ratio I/I^0 vs concentration of 1-PDDA, [Ac]. I^0 is the fluorescence intensity at the initial (highest) acid concentration [Ac]⁰. Excimer fluorescence intensity was measured at 500 nm; excitation at 370 nm; solvent, cyclohexane; temperature, 10 °C. Full line represents the best fit. (b) Dependence of the excimer fluorescence intensity ratio I/I^0 vs molar fraction χ_{Ad} of 9-HDA. Intensity of the excimer fluorescence was measured at 500 nm; excitation at 370 nm; solvent, cyclohexane; temperature, 10 °C.

$$\frac{I}{I^0} = \frac{[Ac_2] + [Ac_2 - Ad]}{[Ac_2]^0} = \frac{[Ac]_f^0(K_1 + K_3K_4[Ad]_f)}{[Ac]^0}$$
(9)

In this equation I^0 and $[Ac_2]^0$ correspond respectively to the excimer intensity and to the concentration of the dimerized acid at the beginning of the experiment before any 9-HDA is added, i.e. when $[Ad]_t = 0$. In a typical experiment, to a solution of 1-PDDA in cyclohexane was added a solution of 9-HDA in the same solvent in volumes calculated to keep the sum of the two concentrations $[Ac]_t$ and $[Ad]_t$ constant throughout the experiment, i.e. $[Ac]_t + [Ad]_t = [C]^0$. Furthermore, if we define the molar fraction of 9-HDA as $\chi_{Ad} = [Ad]_t/[Ac]_t+[Ad]_t$, it turns out that $[Ad]_t = [C]^0 \chi_{Ad}$. By then combining eqs 10 and 11,

$$[Ad]_{t} = [Ad]_{f} + [Ad - Ac] + [Ac_{2} - Ad] + 2[Ad_{2}]$$
(10)

$$[Ac]_{t} = [Ac]_{f} + [Ad - Ac] + 2[Ac_{2} - Ad] + 2[Ac_{2}] \quad (11)$$

which express the balance of masses for the total $[Ac]_{t}$ and $[Ad]_{t}$ concentrations present in the solution at any moment, we have obtained eqs 12 and 13, which express $[Ac]_{f}$ and $[Ad]_{f}$ in terms of K₁, K₂, K₃, K₄, and χ_{Ad} .

TABLE 1

		NMR	excimer fluorescence	
model	equilibrium constant (M^{-1})	25 °C	10 °C	25 °C
$1. \operatorname{Ac} + \operatorname{Ac} \rightleftharpoons \operatorname{Ac}_2$	K1	57 ± 15	11000 ± 1200	4600 ± 700
2. Ac + Ac \rightarrow Ac ₂	K_1	$(57 \pm 15)^{a}$	$(11\ 000\pm 1200)$	(4600 ± 700)
Ac + Ad - Ac - Ad	K_3	130 ± 15	19400 ± 1500	8700 ± 2000
3. $Ac + Ac - Ac_2$	K_1	(57 ± 15)	(11000 ± 1200)	(4600 ± 700)
$Ad + Ad - Ad_2$	K_2	3.1^{b}	233 ^c	100 ^c
Ac + Ad 🕶 Ac-Ad	K_3	(130 ± 15)	$19\ 600\pm 1500$	8800 ± 2000
4. Ac + Ac \Rightarrow Ac ₂	K_1		(11000 ± 1200)	(4600 ± 700)
Ac + Ad 🖚 Ac-Ad	K_3		$19\ 100\pm 1500$	8700 ± 2000
$Ac-Ad + Ac \rightleftharpoons Ac_2-Ad$	K_4		0	0
5. Ac + Ac \Rightarrow Ac ₂	K_1		$(11\ 000\pm 1200)$	(4600 ± 700)
$Ad + Ad - Ad_2$	K_2		233°	100 ^c
$Ac + Ad \Rightarrow Ac - Ad$	K_3		$19\ 600\pm 1500$	8800 ± 2000
$Ac-Ad + Ac - Ac_2 - Ad$	K_4		0	0

^{*a*} Numbers in parentheses are the values, from the present study, used in the calculation of the particular model in order to extract the other parameters. For example, the value 57 ± 15 for K_1 was obtained from the NMR fitting according to model 1 and was then used in the NMR fitting according to model 2 to obtain the value $K_3 = 130 \pm 15$. ^{*b*} From ref 21. ^{*c*} From ref 23.



Figure 7. Dependence of the fluorescence spectra of 10^{-4} M 1-PDDA on the concentration of the added 9-HDA. (A) [9-HDA] = 0; (B) 4.9 $\times 10^{-5}$ M; (C) 8.7 $\times 10^{-5}$ M; (D) 1.22×10^{-4} M; (E) 1.96×10^{-4} M; (F) 2.62×10^{-4} M. The isoemissive point at ca. 437 nm is evident. Excitation of all solutions was at 370 nm.

$$2K_{2}[\mathrm{Ad}]_{f}^{2} + [\mathrm{Ad}]_{f}(1 + K_{3}[\mathrm{Ac}]_{f} + K_{3}K_{4}[\mathrm{Ac}]_{f}^{2}) - [\mathrm{C}]^{0}\chi_{\mathrm{Ad}} = 0 \quad (12)$$
$$2K_{1}[\mathrm{Ac}]_{f}^{2} + [\mathrm{Ac}]_{f}(1 - K_{3}[\mathrm{Ad}]_{f}) - 4K_{2}[\mathrm{Ad}]_{f}^{2} - 2[\mathrm{Ad}]_{f} + [\mathrm{C}^{0}](3\chi_{\mathrm{Ad}} - 1) = 0 \quad (13)$$

To fit eq 9 to the experimental data, i.e. I/I^0 and χ_{Ad} , we have used eqs 6, 12, and 13 as well as the previously determined values of K_1 and the value of K_2 found in the literature²³ for the self-association of 9-ethyladenine. The best values obtained from these fittings were $K_3(10 \text{ °C}) = (1.96 \pm 0.15) \times 10^4 \text{ M}^{-1}$, $K_3(25 \text{ °C}) = (8.8 \pm 2) \times 10^3 \text{ M}^{-1}$, $K_4(10 \text{ °C}) = 0$ and $K_4(25 \text{ °C}) = 0$, and they are listed in Table 1 (model 5). These results indicate that only two complexes are formed in solutions of cyclohexane, namely, the dimerized 1-PDDA and the 1:1 complex between 1-PDDA and 9-HDA, which are in equilibrium with the uncomplexed monomers of 1-PDDA and 9-HDA.

In a similar study of the H-bonding interactions between 9-ethyladenine and butyric acid in cyclohexane, using the method of differential absorption spectroscopy,²³ it was found that up to a value of ca. 8 for the ratio [butyric acid]/ [9-ethyladenine] the equilibrium constant $K_{1:1}$ for the formation of a 1:1 complex between the two reactants was $(2.2 \pm 0.4) \times$ 10^4 M^{-1} , which is in good agreement with our value $K_3 = (1.96 \text{ M}^{-1})$ \pm 0.15) \times 10⁴ M⁻¹. However, at higher values of this ratio a different model was proposed²³ according to which, in addition to the 1:1 dimers, trimers made of two acid units and one base were also formed with $K_{2:1} = (2.0 \pm 0.4) \times 10^3 \text{ M}^{-1}$ and $K_{1:1}$ = $(2.7 \pm 0.4) \times 10^4 \text{ M}^{-1}$. Contrary to these reports, our results do not support the existence of any trimers in the solution. Indeed, when model 4 was used to fit the experimental data (Figure 6b), the fitting was excellent for $K_4 = 0$ even at the lowest values of the molar fraction χ_{Ad} , where the highest acid concentration occurs and the population of trimers is favored. In addition, computer simulations in which values of $(1-3) \times$ 10^3 M^{-1} were introduced for the constant K_4 were very poor compared to similar simulations with $K_4 = 0$. Further evidence that trimers do not form in these solutions was provided by the appearance of an isoemissive point around 437 nm in the fluorescence spectra of 1-PDDA as 9-HDA was added (Figure 7). An isoemissive point, under the present experimental conditions, i.e. constant absorption of the exciting light, indicates the competition of two different species for the monomer and excimer fluorescence. Indeed, these species are the Ac₂ dimer, which forms excimers, and the Ac, either as free monomer or as the one partner in the dimers Ac-Ad, which emits monomeric fluorescence. Should trimers of the type Ac₂-Ad or any higher complexes form in these solutions, the isoemissive point would have been lost since such complexes would constitute an additional source of excimer fluorescence.

Very nearly identical values for the equilibrium constants, estimated from the most general model 5, have been obtained also from the partial models 2–4 (Table 1). Thus, model 2, which takes into account only the complexes Ac-Ac and Ac-Ad while it assumes the Ad-Ad dimerization to be negligible, produces (after introducing in the fitting the already known values of K_1) for K_3 the values $K_3(10 \text{ °C}) = (1.94 \pm 0.15) \times 10^4 \text{ M}^{-1}$ and $K_3(25 \text{ °C}) = (8.7 \pm 0.2) \times 10^3 \text{ M}^{-1}$. These values of K_3 were not affected when, in addition to the values of K_1 , values for K_2 found in the literature,²³ between 1×10^2 and $3 \times 10^2 \text{ M}^{-1}$, were introduced and the fitting was performed according to model 3, which takes into account the complexes Ac-Ac, Ac-Ad, and Ad-Ad (Table 1). Fittings according to

model 4, which involves acid self-association Ac-Ac, the Ac-Ad dimerization, and Ac₂-Ad trimer formation, where the values of K_1 and K_2 were used as in model 3, gave for K_3 the values $(1.91 \pm 0.15) \times 10^4$ M⁻¹ at 10 °C and $(8.7 \pm 0.2) \times$ 10^3 M⁻¹ at 25 °C, whereas the trimerization constant K_4 turned out to be equal to 0 at both temperatures. In conclusion, all evidence indicates that in cyclohexane, where the association of the reactants is quite strong, at least compared to chloroform, only Ac-Ac and Ac-Ad dimers are formed in adequate proportions, while Ad-Ad dimerization is extremely feeble. These results also confirm the previously discussed explanation for the observed deviation from the symmetric shape of the Job plot (Figure 4), viz., that it is due to the formation of the two complexes Ac-Ac and Ac-Ad and not to any higher complexes.

IV. Conclusions

The main conclusions of the present work, on the one hand, constitute a clear demonstration of the importance of fluorescence spectroscopy for studies of molecular recognition and, on the other, concern the applications of these methods to the study of molecular recognition. Indeed, it has been proved that fluorescence spectroscopy can be successfully applied to the study of molecular recognition, either as complementary to other methods or by itself. In a combined NMR and fluorescence study the predominant equilibria between 1-PDDA and 9-HDA in chloroform and cyclohexane were established and the corresponding constants were determined with accuracy. The overall equilibrium involves only dimerizations, the stronger being the Ac-Ad complexation; the Ac-Ac self-association is ca. 40% weaker, whereas the formation of Ad-Ad dimers is extremely weak. Trimer formation, though previously reported in the literature, was not confirmed by the present study. The equilibrium constants have considerably smaller values in solvents which can themselves participate in H-bonding than in inert solvents, the differences measuring up to more than 3 orders of magnitude.

References and Notes

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