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INVESTIGATIONS ON PURINE AND PYRIMIDINE BASES STACKING ASSOCIATIONS IN AQUEOUS SOLUTIONS BY THE FLUORESCENCE QUENCHING METHOD. III. INTRAMOLECULAR ASSOCIATION OF 9,9'-[1,3-PROPYLENE]-BIS-2-AMINOPURINE

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General equations relating fluorescence quantum yield and lifetime of a compound with its intramolecular stacking equilibrium and kinetics were derived. Intramolecular stacking association of 9,9'-[1,3-propylene]-bis-2-aminopurine in aqueous solution was examined within the range of temperatures from 0 to 90°C. A two-state thermodynamic model of the association was verified. The stacking enthalpy and entropy can be taken, with a good approximation, as temperature-independent ($\Delta H = -2.0$ kcal/mol, $\Delta S = -3.25$ e.u.) although the function $\Delta G = -0.00886\,T^2 + 8.847\,T - 2876$ describes more precisely the observed changes of stacking free enthalpy with temperature. The association rate constants were determined. Activation energy of the reaction (2 kcal/mol) is the same as in the case of association between free 2-aminopurine molecules. It confirms a two-step mechanism of the process. The advantages and shortcomings of the fluorescence quenching method are discussed.

1. Introduction

In previous parts of this work, application of the fluorescence quenching method to investigations of stacking auto- [1] and heteroassociations [2] has been presented. The present paper concerns intramolecular stacking of such molecules as dinucleotides and their model compounds.

Some studies in this field have already been made. The equilibrium constants of intramolecular stacking between indole and nucleobase residues linked by a propylene bridge have been determined lately by Mutai et al. [3] from indole fluorescence measurements. Intramolecular stacking equilibrium in dinucleotides and their model compounds, containing 1,N^6-ethenoadenine moiety as a fluorescent probe, was investigated by several authors [4–6].

In the present paper processes responsible for internal quenching are considered. On this basis we formulate the general principles of the fluorescence quenching method as applied to studies of intramolecular stacking associations. The possibilities and limitations of the method as well as practical problems of its application are discussed. By using this method investigations of intramolecular stacking equilibrium and kinetics of a model compound, 9,9'-[1,3-propylene]-bis-2-aminopurine, were performed.

2. Theoretical

The object of our considerations will be a molecule Z with two chromophores X and Y. Only two thermodynamically different states of the molecule occur: the stacked $\overset{X}{Y}$ and the unstacked form $X\_\_\_Y$. The stacking equilibrium is thus described by a constant $K = C_s/C_u$; where C_s and C_u denote the concentrations of both forms. Formation of intramolecular exciplexes is postulated. They can be formed either directly via excitation of the stacked molecule, or by excitation of the unstacked molecule followed by reorientation of the chromophores. Our aim was to find an expression describing the fluorescence quantum yield of chromophore X.

Let ϵ_X, ϵ_Y and ϵ_s denote the extinction coefficients of unstacked chromophores and stacked molecule Z at the excitation wavelenght. Hypochromicity due to stacking is thus described as follows:

$$\alpha = \epsilon_s/(\epsilon_X + \epsilon_Y). \quad (1)$$

The measured extinction coefficient of compound Z is:

$$\epsilon_{obs} = \frac{(\epsilon_X + \epsilon_Y)\, C_u + \epsilon_s C_s}{C_u + C_s} = \frac{\epsilon_X + \epsilon_Y + \epsilon_s K}{1 + K} \quad (2)$$

and the observed hypochromic coefficient:

$$\alpha_{obs} = \frac{\epsilon_{obs}}{\epsilon_X + \epsilon_Y} = \frac{1 + \alpha K}{1 + K}. \quad (3)$$

Reactions leading to formation and decay of the excited chromophores X along with their rates are listed below. The intensity of light absorbed by a solution of Z is expressed by I_{abs} mol/ℓ s and concentration of the solution is denoted by $C = C_s + C_u$.

Reaction			Reaction rate
X–Y	$\xrightarrow{h\nu}$	X^*–Y	$I_{abs}\frac{\epsilon_X C_u}{\epsilon_{obs} C}$
$\binom{X}{Y}$	$\xrightarrow{h\nu}$	$\binom{X}{Y}^*$	$I_{abs}\frac{\epsilon_s C_s}{\epsilon_{obs} C}$
X^*–Y	$\longrightarrow$	X–Y $+ h\nu$	$[X^*]\, k_\varphi$
X^*–Y	$\longrightarrow$	X–Y	$[X^*]\, k_n$
X^*–Y	$\longrightarrow$	$\binom{X}{Y}^*$	$[X^*]\, k_e$
$\binom{X}{Y}^*$	$\longrightarrow$	$\binom{X}{Y} + h\nu$	$[E]\, k_{\varphi e}$
$\binom{X}{Y}^*$	$\longrightarrow$	$\binom{X}{Y}$	$[E]\, k_{ne}$
$\binom{X}{Y}^*$	$\longrightarrow$	X^*–Y	$[E]\, k_{de}$

For the sake of simplification the following process:

$$X\text{–}Y \xrightarrow{h\nu} X\text{–}Y^* \longrightarrow \binom{X}{Y}^* \longrightarrow X^*\text{–}Y$$

leading to chromophore X excitation has been neglected with the assumption that either the lifetime of the excited chromophore Y^* is very short, or the probability of exciplex dissociation is low. Accordingly, exciplex dissociation $\binom{X}{Y}^* \longrightarrow X\text{–}Y^*$ can also be neglected as not involved in chromophore X fluorescence. For more general treatment of the problem see ref. [7].

Besides, the probability p of exciplex formation after excitation of the stacked molecule is believed to be equal to unity and resonance energy transfer between the chromophores has been excluded. The latter assumptions have been discussed at length previously [1].

In the steady-state the following equations are fulfilled:

$$I_{abs}\frac{\epsilon_X C_u}{\epsilon_{obs} C} + [E]\, k_{de} = [X^*]\,(k_\varphi + k_n + k_e) \quad (4)$$

$$I_{abs}\frac{\epsilon_s C_s}{\epsilon_{obs} C} + [X^*]\, k_e = [E]\,(k_{\varphi e} + k_{de} + k_{ne}) \quad (5)$$

From eqs. (2), (4) and (5) one obtains:

$$\frac{k_\varphi\,[X^*]}{I_{abs}} = \frac{\epsilon_X/(\epsilon_X + \epsilon_Y) + \alpha\gamma K}{1 + \alpha K}\,\frac{k_\varphi}{k_\varphi + k_n + (1-\gamma)k_e}, \quad (6)$$

where γ is the probability of exciplex dissociation:

$$\gamma = k_{de}/(k_{de} + k_{\varphi e} + k_{ne}). \quad (7)$$

Let us use the following notations:

$$\Phi_m = k_\varphi/(k_\varphi + k_n) \quad \text{and} \quad \tau_m = 1/(k_\varphi + k_n). \quad (8)$$

The left-hand side of eq. (6) is equal to the fluorescence quantum yield Φ of chromophore X. Then:

$$\Phi_m/\Phi = [1 + (1-\gamma)\,\tau_m\, k_e]\,\frac{1 + \alpha K}{\epsilon_X/(\epsilon_X + \epsilon_Y) + \alpha\gamma K}. \quad (9)$$

When X = Y one obtains in a similar way:

$$\Phi_m/\Phi = [1 + (1-\gamma)\,\tau_m\, k_e]\,\frac{1 + \alpha K}{1 + \alpha\gamma K}. \quad (10)$$

In this case no assumptions, except for $p = 1$, are necessary.

Eqs. (9) and (10) are analogous to those found previously in the case of auto- [1] and heteroassociations [2]. The right-hand side of each of them is a product of two terms: the static term

$$S\,\frac{1 + \alpha K}{\epsilon_X/(\epsilon_X + \epsilon_Y) + \alpha\gamma K} \quad \text{or} \quad S = \frac{1 + \alpha K}{1 + \alpha\gamma K} \quad (11)$$

equal to the reciprocal of the excitation quantum yield of chromophore X, and the dynamic term:

$$D = 1 + (1-\gamma)\,\tau_m\, k_e \quad (12)$$

equal to the ratio of lifetimes $D = \tau_m/\tau$, where τ, the lifetime of the excited chromophore X^* is defined by

the equation ‡:

$$\tau = \frac{1}{k_\varphi + k_n + (1-\gamma)k_e}. \quad (13)$$

It is always possible to find such a compound the rate constants k_φ and k_n of which are the same as those of chromophore X. It is an appropriate nucleoside when Z is a dinucleotide, and a 9-alkyl derivative of the base in the case of model compounds e.g. 2AP-C_3-2AP. Therefore, according to eq. (8), Φ_m and τ_m values can be found experimentaliy. The dynamic term $D = \tau_m/\tau$ can be measured as well. If only the extinction coefficients and α are known, and parameter γ can be estimated, the equilibrium constant K can be calculated from eqs. (9) or (10), respectively.

It should be stressed here that our analysis is valid and its results are applicable only if a two-state model of association holds true. That is when only one associated and one dissociated form of the molecule occur both in the ground and in the excited state of the molecule. Each of the forms may correspond to several relative orientations of chromophores X and Y, or to a quasi-continuum of such orientations, but an energy barrier for rearrangement of the chromophores is at most of the same order of magnitude as kT.
As far as the stacked form is concerned, the applicability of this model cannot be verified directly by fluorescence measurements. Nevertheless, thermodynamically different unstacked forms can be discerned by fluorescence decay measurements [8]. A single fluorescence lifetime proves that the exciplex formation rate constant k_e is the same for all unstacked molecules, so the Boltzmann distribution of energy is realized.

If $\gamma = 0$ and $\epsilon_Y = 0$, eqs. (9) and (10) take the form corresponding to the relationships used so far [3–6] in fluorescence quenching analysis.

‡ If $K \neq 0$ and $\gamma \neq 0$, the fluorescence decay is not exponential. Nevertheless, the Stern–Volmer quenching law may be fulfilled, and accordingly τ values determined from fluorescence quenching measurements [7].

3. Experimental

3.1. Materials and methods

9,9'-[1,3-propylene]-bis-2-aminopurine (2AP-C_3-2AP) was synthesized by the method described by Browne et al. [9]. 9-Ethyl-2-aminopurine (Et-2AP) was a Cyclo Chemical Co. product. Both compounds were purified by repeated thin-layer chromatography on silica gel followed by crystallization from ethanol. Purity of the materials was checked by comparison of their absorption and emission spectra as well as fluorescence quantum yields before and after the last rechromatography. M.p. of 2AP-C_3-2AP was 335°C.

The fluorescence signals of aqueous solutions of Et-2AP and 2AP-C_3-2AP were measured at the apparent maximum of their fluorescence spectra, 370 nm, with excitation at 304 nm, close to the maxima of their absorption spectra: 303.5 nm and 302 nm. The apparatus and method of measurements were described elsewhere [10].

Extinction of the solutions was measured at the excitation wavelength with a Cary-118 spectrometer. An interference filter with transmission maximum at 304 nm was put behind the samples to cut-off their fluorescence light.

The fluorescence lifetimes were measured with a phase fluorometer in the Institute of Physics, Polish Academy of Sciences, Warsaw.

3.2. Results and discussion

Excimer fluorescence of 2AP-C_3-2AP is not observed at temperatures above 0°C. The fluorescence spectrum of the compound is close to that of Et-2AP. The absorption spectra are also very similar. Only a small (1.5 nm) batochromic shift of the Et-2AP spectrum is observed. Consequently, the same rate constants k_φ and k_n can be expected for both compounds. Et-2AP is thus a good reference compound for determination of Φ_m and τ_m values.

The fluorescence quenching of 2AP-C_3-2AP by bromide anions was examined at 20.1°C. The Stern–Volmer law was fulfilled and the fluorescence spectrum remained unchanged up to 1 mol/l of KBr concentration. It proves that at all wavelength the fluorescence decay is characterized by the same, single lifetime. Thereby, the existence of only one dissociated

form of the molecule was established. We could not ascertain whether only one stacked form occurs as well. Nevertheless, the results presented below seem to confirm indirectly the two-state model on which analysis of data was based.

a) Determination of the dynamic factor D and stacking rate constants k_e.

Fluorescence signals of diluted aqueous solutions of 2AP-C_3-2AP and Et-2AP with and without KBr were measured at nineteen temperatures within the range 0.6 to 88.4°C. The optical densities of all samples were about 0.06/mm. KBr concentration C_q = 1 mol/ℓ. The quenching constants $\kappa_q^m = k_q\tau_m$ and $\kappa_q = k_q'\tau$ were calculated by the Stern–Volmer equation:

$$\Phi_0/\Phi = 1 + \kappa_q C_q. \tag{14}$$

The fluorescence lifetime of Et-2AP was measured at 24°C. For other temperatures the lifetimes were calculated from measured fluorescence quantum yields. Thus, it was possible to calculate the rate constants k_q of the compound.

Analysis of the reaction kinetics [7] provides strong premisses for the supposition that the quenching rate constants for both compounds, k_q and k_q', are close to one another. Direct lifetime measurements fully confirmed these expectations. The ratio of lifetimes measured at 24°C was, within the limits of measurement error like that of κ values: τ_m/τ = 9.13, whereas κ_q^m/κ_q = 9.04.

The calculated values of the dynamic factor $D = \kappa_q^m/\kappa_q$ are given in the second column of table 1.

From eqs. (13) and (8) one obtains:

$$k_e = \frac{D-1}{(1-\gamma)\tau_m}. \tag{15}$$

Under the assumption that $\gamma = 0$, the excimer formation rate constants k_e were calculated (table 1, column 3). The rate constant of an intramolecular reaction can be described formally by a similar function of temperature as a rate constant of a reaction between free molecules:

$$k_e = \text{const.}'(T/\eta)\left\{1 + \frac{\text{const.}'\, T/\eta}{\text{const.}''\sqrt{T}\exp(-\Delta E/RT)}\right\}^{-1}. \tag{16}$$

After establishing the activation energy ΔE by iterative procedure and calculation of the parameters const.' and const.'' by the least-squares method, a very good fit of function (16) to the experimental k_e^{exp} values was obtained. Thereby, the correctness of the assumption $\gamma = 0$ was confirmed. The interpolated values of the excimer formation rate constants k_e^{int} are given in table 1 (column 4), along with the deviations of the experimental points from the interpolation curve (column 5). The D^{int} values calculated from the interpolated k_e^{int} constants are given in the last column of table 1. They were used in further calculation.

Table 1
Dynamic factors D of 2AP-C_3-2AP fluorescence quenching and intramolecular excimer formation rate constants k_e

Temp. (°C)	D^{exp}	k_e^{exp} ($10^8\,s^{-1}$)	k_e^{int} ($10^8\,s^{-1}$)	Δk_e^{exp} (%)	D^{int}
0.6	5.74	3.78	3.90	−3.1	5.89
5.5	6.65	4.53	4.43	2.3	6.53
10.4	7.20	5.01	5.01	0.1	7.20
15.2	7.87	5.60	5.58	0.3	7.85
20.1	8.57	6.24	6.19	0.7	8.52
25.0	9.20	6.83	6.82	0.1	9.19
29.9	9.88	7.50	7.47	0.4	9.85
34.8	10.53	8.17	8.15	0.3	10.50
39.6	11.21	8.90	8.82	0.8	11.12
44.5	11.82	9.61	9.53	0.8	11.74
49.4	12.30	10.24	10.27	−0.2	12.32
54.3	12.88	11.02	11.02	0.0	12.88
59.1	13.11	11.52	11.78	−2.2	13.39
64.0	13.77	12.48	12.57	−0.8	13.87
68.9	14.17	13.24	13.38	−1.0	14.31
73.8	14.66	14.18	14.21	−0.3	14.70
78.6	15.10	15.13	15.05	0.5	15.03
83.5	15.39	16.00	15.92	0.5	15.32
88.4	15.71	16.98	16.81	1.0	15.56

exp - experimental; int - calculated from eq. (16) for ΔE = 2 kcal/mol.

For ΔE = 2.0 kcal/mol the fit of function (16) was the best. Exactly the same value of the activation energy was obtained in the case of 2-AP excimer formation [1] and a very similar one (ΔE = 2.7 kcal/mol) for 2-AP + thymidine exciplex formation [2]. This confirms the two-step mechanism of the reaction proposed by Pörschke and Eggers [11] and discussed in the first part of this work [1]. According to it, the activation energy depends mainly on the solvent properties and is expected to be very similar for all associating species, if only the solute-solvent interaction

energies are not dramatically different. Therefore, the activation energies of association in the ground and excited states of the molecule are very similar. So are the frequency factors of the reaction and diffusion coefficients of the reacting species. The k_e values can thus be considered as the 2AP-C_3-2AP intramolecular stacking rate constants.

b) Determination of stacking equilibrium constants K.

The optical densities of the investigated samples were very similar, and low enough to presume that the measured fluorescence signals V were proportional to the absorbance. In such a case, a more convenient form of eq. (10) can be obtained after simple transformations. If $\gamma = 0$,

$$\frac{V_m}{V}\,\frac{A'}{A'_m} = D(1+K)\,\alpha'_{obs}, \qquad (17)$$

where A' and A'_m are absorbances, and α'_{obs} the observed hypochromic coefficient at 20°C.

The latter value was deduced from a comparison of the extinction coefficients of several 2-AP derivatives in aqueous and ethanolic solutions. For 2-AP, Et-2AP, N,N-dimethyl-9-methyl-2AP and N-methyl-9-methyl-2AP the ratio of the extinction coefficients in ethanol and water is the same and equals 1.01, whereas in the case of 2AP-C_3-2AP it is 1.21. This phenomenon can be attributed only to the intramolecular stacking of molecules, when dissolved in watet, leading to a decrease of their extinction coefficient. The calculated hypochromic coefficient $\alpha'_{obs} = 0.843$.

The equilibrium constants K calculated from eq. (17) are presented in table 2, column 2. The Van't Hoff linear relationship $\ln K = f(1/T)$ is fairly well fulfilled (see fig. 1). $\Delta H = -2.00$ kcal/mol and $\Delta S = -3.25$ e.u. The interpolated K values and deviations of the experimental points from the interpolation curve are given in columns 3 and 4 of table 2. In columns 5 and 6 the results of interpolation when using the second-order relationship:

$$\Delta G = -0.00886\,T^2 + 8.847\,T - 2876 \qquad (18)$$

are shown. This function, corresponding to the dashed line in fig. 1, fits better the experimental points. The thermodynamical parameters of the association seem thus to change slightly with temperature in a similar way as those of 2-AP dimerization [1].

The hypochromic effect of 2AP-C_3-2AP intramolecular association could be calculated from eq. (3). Its value $\alpha = 0.816$, much lower then in the case of 2-AP autoassociation [1], seems to indicate that the relative orientations of the stacked molecules are different in both cases.

Table 2
2AP-C_3-2AP intramolecular stacking equilibrium constants

Temp. (°C)	K^{exp}	$^{+}K^{int}$	$^{+}\Delta K^{exp}$ (%)	$^{++}K^{int}$	$^{++}\Delta K^{exp}$ (%)
0.6	7.83	7.67	2.0	7.80	0.3
5.5	7.32	7.19	1.8	7.27	0.7
10.4	6.82	6.76	0.9	6.79	0.4
15.2	6.35	6.37	−0.4	6.37	−0.4
20.1	5.92	6.01	−1.5	5.99	−1.2
25.0	5.61	5.68	−1.3	5.65	−0.7
29.9	5.29	5.38	−1.6	5.34	−0.8
34.8	5.06	5.10	−0.8	5.05	0.1
39.6	4.85	4.85	−0.1	4.80	0.9
44.5	4.62	4.62	0.0	4.57	1.0
49.4	4.39	4.40	−0.3	4.36	0.6
54.3	4.19	4.20	−0.3	4.17	0.5
59.1	3.94	4.02	−2.0	3.99	−1.4
64.0	3.83	3.84	−0.4	3.83	0.0
68.9	3.69	3.68	0.1	3.68	0.1
73.8	3.55	3.53	0.5	3.55	0.2
78.6	3.43	3.40	1.0	3.42	0.2
83.5	3.30	3.27	1.1	3.31	−0.1
88.4	3.19	3.14	1.5	3.20	−0.2

exp - experimental; int - calculated from the equations: (+) $\Delta G = 3.25\,T - 2000$, (++) $\Delta G = -0.00886\,T^2 + 8.847\,T - 2876$.

In a 2AP-C_3-2AP molecule, the number of allowed relative orientations of the chromophores is limited because of steric hindrance. The realized orientations correspond, evidently, to strong interactions between transition moments of the chromophores, since an excimer of low dissociation probability can be formed. Hence, a stronger hypochromic effect is observed than in the case of free molecules association, where a much greater variety of chromophore orientations occurs.

4. Conclusions

The fluorescence quenching method seems to be particularly promising when applied to intramolecular stacking investigations. Its precision in determination of stacking equilibrium and kinetics is beyond com-

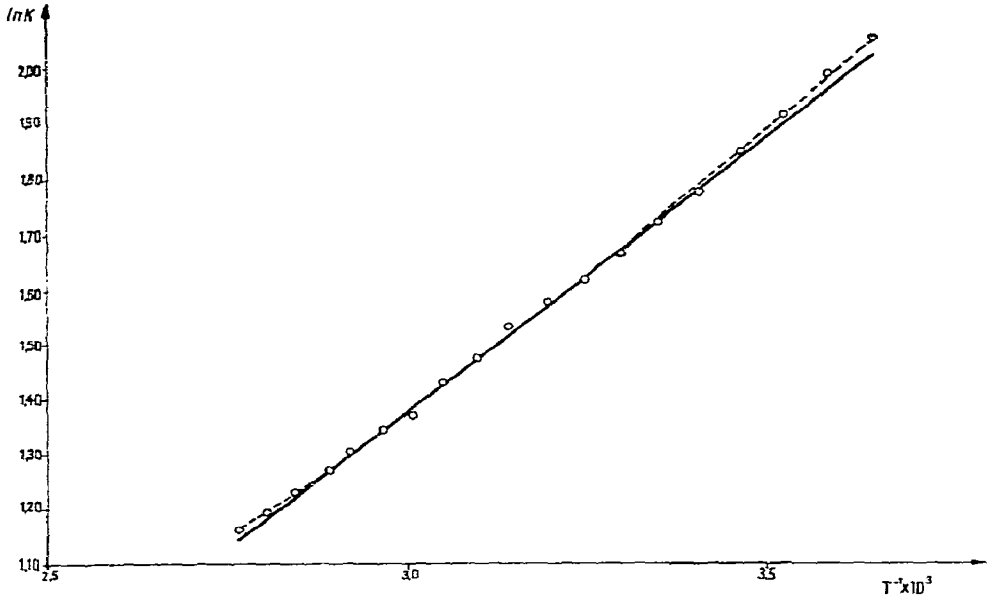

Fig. 1. Van't Hoff plots of 2AP-C_3-2AP intramolecular stacking equilibrium constant. The full line corresponds to $\Delta H = -2.00$ kcal/mol and $\Delta S = -3.25$ e.u. The dashed line was calculated from the equation $\Delta G = -0.00886\,T^2 + 8.847\,T - 2876$.

parison with any other method used so far. Analysis of fluorescence decay provides a deeper insight into the conformational structure of the compound, namely it allows to discern different thermodynamic states of the unstacked form of the molecule.

5. Limitations of the fluorescence quenching method

Three conditions have to be fulfilled by a compound to be investigated by this method: 1) its fluorescence must be quenched by the association, 2) its fluorescence quantum yield must be high enough to be measured with a sufficient accuracy, and 3) its fluorescence lifetime should be longer than 0.1 ns.

Among aromatic and heterocyclic compounds excimer and exciplex formation is a common phenomenon. The first condition is, hence, usually fulfilled. Still, there are some purine derivatives, such as the Y base, and several dyes the fluorescence of which is not quenched, but on the contrary enhanced by association. Stacking properties of those compounds are examined fluorometrically, but of course not by the method here described.

If exciplex emission is observed, separation of the monomer and exciplex fluorescence bands can always be done, and analysis of both fluorescence quantum yields done independently. Then, the results obtained from monomer fluorescence quenching may be compared and confirmed by those derived from exciplex fluorescence measurements.

No serious limitations arise from the second condition. The measurement techniques are so well developed nowadays that even extremely low fluorescence signals can be determined with satisfactory precision. By the single-photon counting method, the fluorescence of natural nucleic-acid basis ($\Phi < 10^{-4}$) is easily measurable [12].

The most limiting condition is the third one. If fluorescence lifetime is shorter than 0.1 ns one cannot be sure that the exciplex formation probability p is equal to 1 (see basic assumptions in part I [1]), and it is difficult or even impossible to determine its actual value. Unfortunately, this difficulty has to be envisaged in the case of all natural nucleic bases, apart from Y base. Nevertheless, a great deal of valuable information on the stacking properties of nucleic bases can be obtained by using their fluorescent model compounds, such

as 2-AP and 2-aminopyrimidine. As the stacking rate constants are only slightly dependent on the chemical structure of associating molecules, the stacking kinetics can be fully examined in this way.

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