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INVESTIGATIONS ON PURINE AND PYRIMIDINE BASES STACKING ASSOCIATIONS IN AQUEOUS SOLUTIONS BY THE FLUORESCENCE QUENCHING METHOD. I. AUTOASSOCIATION OF 2-AMINOPURINE

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A general equation was derived, describing fluorescence quantum yield and lifetime of an autoassociating compound in liquid solutions. The autoassociation of 2-aminopurine in aqueous solution was examined within the range from 0 to 90°C. The compound seemed to associate cooperatively. The thermodynamic parameters of polymerization change with temperature, so that its free enthalpy $\Delta G = -0.0797\ T^2 + 45.4\ T - 7893$. The dimerization enthalpy and entropy are approximately temperature-independent ($\Delta H_2 = -4.17$ kcal/mol, $\Delta S_2 = -10.9$ e.u.), although the function: $\Delta G_2 = -0.0308\ T^2 + 30.3\ T - 7213$ fits experimental points better. The observed dependences can be explained by the increasing role of the hydrophobic effect with temperature and size of the aggregates. The association rate constants were determined, and a two-step reaction mechanism was demonstrated. The first step is diffusion-controlled. The second is characterized by an activation energy of ~2 kcal/mol and an encounter distance of ~8.3 Å.

1. Introduction

Stacking interactions between nucleobases are one of the crucial factors determing the secondary structure of nucleic acids and the dynamics of their conformational transitions. Unfortunately, in spite of theoretical and experimental efforts of the last 14 years [1], the phenomenon is far from being satisfactorily known. The nature of association forces remains still unclear. Information concerning internal stacking equilibrium in dinucleotides and single-stranded polynucleotides as well as association kinetics is scarce and unprecise. Development of experimental techniques (vapour-pressure osmometry) permitted relatively accurate studies of the autoassociation equilibrium of free molecules. But even in this case, the description of the stacking equilibrium has usually to be restricted to a rather oversimplified form based on the isodesmic model of association,

Further progress in stacking investigations seems to depend mainly on the application of new experimental techniques. One of them could be the fluorescence quenching method. Relative fluorescence quantum yields of solutions can be measured with high accuracy within a broad range of concentrations [2]. Quenching and selfquenching phenomena are well known, and their theory is well developed [3].

Several processes may be responsible for the drop of fluorescence quantum yield and concomitant shortening of fluorescence lifetime, such as: excitation energy transfer, excimer or exciplex formation, electron exchange. Independently of its nature, fluorescence quenching is always enhanced by association of molecules, but fluorescence lifetime is usually not shortened. Therefore stacking equilibrium can be determined from the fluorescence quenching measurements. Besides, some valuable information concerning kinetics of stacking process can be obtained in this way.

The first attempts in this direction have already been undertaken. Fluorescence quenching has been used recently as a measure of intramolecular stacking in dinucleotides and their model compounds [4–7]. An attempt was also made to estimate, in this way, the equilibrium constants of association between free molecules [8]. Nevertheless, no systematic analysis of the processes underlying the phenomenon has been published so far.

The aim of the present work, preliminary results of which were communicated for the first time a year ago [9], was to formulate the theoretical bases of the fluorescence quenching method and to determine the possibilities of its practical application. In three, consecutive parts of the paper the principles of the method, as applied to investigation of auto- (this part) and heteroassociation [10] of free molecules and of intramolecular stacking [11] are formulated and its possibilities and limitations are discussed.

As far as pyrimidine and purine bases are concerned, excimer formation is responsible, most probably, for quenching [12,13]. Hence, our theoretical treatment is based on this assumptions. Nevertheless, its mathematical formalism remains the same for any other quenching process in which a physical contact between molecules is involved, and so it is applicable in all cases, apart from the case of resonance energy transfer.

2-Aminopurine (2-AP) and its derivatives have been chosen to verify the applicability of the method. The spectroscopic properties of 2-AP are well known [14]. Its high fluorescence quantum yield and long lifetime facilitate the measurements. Besides, 2-AP can sometimes be used as a fluorescent conformational probe [15,16], so the relationship between its emission properties and stacking is of special interest.

2. Theoretical

For partly associated molecules two ways of excimer formation are possible: a diffusion-dependent reaction between excited and unexcited molecules and excitation of a molecule stacked in its ground state. The following basic assumptions are made:

1) Excitation of a stacked molecule leads with probability $p = 1$ to excimer formation.

This assumption holds true if the relative orientation of the associated molecules is the same in their ground and excited states, or if a necessary reorientation is fast as compared with the natural lifetime of the excited molecule. Separation of the molecules before excimer formation may be neglected because it is a relatively slow process as compared with the reorientation movement. For such molecules as nucleobases, the reorientation time is estimated to be of the order of a few picoseconds, so that p is always close to unity for fluorescence lifetimes longer than 0.1 ns.

2) Even if excimers of different geometry are formed, the probability of dissociation, γ, is equal for all of them.

3) The resonance transfer of the excitation energy may be neglected.

The probability of this latter process can be estimated from spectroscopic data of the investigated compounds [17]. For free nucleobases, nucleosides and their model compounds, it is usually close to zero because of either a small overlap of the absorption and emission spectra or a shoft fluorescence lifetime of molecules [13]. If necessary, a full description of quenching, taking into account energy transfer as well, is possible. The problem has been discussed recently by Bojarski [18,19].

According to assumptions 1–3, all excitation and relaxation processes, together with their rates, can be described as follows:

Reaction	Reaction rate
$M \xrightarrow{h\nu} M^*$	$I_{abs}\frac{C_m}{\beta + C_m}$
$M_n \xrightarrow{h\nu} E$	$I_{abs}\frac{\beta}{\beta + C_m}$
$M^* \rightarrow M + h\nu$	$k_\varphi[M^*]$
$M^* \rightarrow M$	$k_n[M^*]$
$M^* + M_i \rightarrow E$	$[M^*]\sum_{i=1}^{\infty} k_e^i[M_i]$
$E \rightarrow M_n$	$k_{ne}[E]$
$E \rightarrow M_n + h\nu$	$k_{\varphi e}[E]$
$E \rightarrow M^* + M_i$	$k_{de}[E]$

Symbols used: M monomer molecule, M_n, M_i aggregates of n or i molecules, where $n \geqslant 2$ and $i \geqslant 1$, $C_m = [M]$, E excimer, I_{abs} light absorbed by 1ℓ of solution in moles of quanta per second, $\beta = \Sigma_{n=2}^{\infty} n\alpha_n[M_n]$, where α_n parameters describe hypochromicity due to association: $\alpha_n = \epsilon_n/\epsilon$. ϵ and ϵ_n denote the extinction coefficients, at the excitation wavelength, of monomers and molecules stacked in M_n aggregates, respectively. k_n, k_φ, k_e^i, k_{ne}, $k_{\varphi e}$ and k_{de} are rate constants of the corresponding processes.

In steady-state the following relationship are fulfilled:

$$I_{\text{abs}} \frac{C_{\text{m}}}{C_{\text{m}} + \beta} + [\text{E}]\, k_{\text{de}} = [\text{M}^*]\,(k_{\varphi} + k_n + \sum_{i=1}^{\infty} k_{\text{e}}^i [\text{M}_i]) \quad (1)$$

and

$$I_{\text{abs}} \frac{\beta}{C_{\text{m}} + \beta} + [\text{M}^*] \sum_{i=1}^{\infty} k_{\text{e}}^i [\text{M}_i] = [\text{E}]\,(k_{\text{ne}} + k_{\varphi\text{e}} + k_{\text{de}}). \quad (2)$$

Therefrom:

$$\frac{k_{\varphi}[\text{M}^*]}{I_{\text{abs}}} = k_{\varphi}\left\{\frac{C_{\text{m}}}{C_{\text{m}} + \beta} + \frac{k_{\text{de}}}{k_{\text{ne}} + k_{\varphi\text{e}} + k_{\text{de}}} \frac{\beta}{C_{\text{m}} + \beta}\right\} \times \left\{k_{\varphi} + k_n + \left(1 - \frac{k_{\text{de}}}{k_{\text{ne}} + k_{\varphi\text{e}} + k_{\text{de}}} \sum_{i=1}^{\infty} k_{\text{e}}^i [\text{M}_i]\right)\right\}^{-1} \quad (3)$$

The left hand side of eq. (3) is the monomer fluorescence quantum yield Φ. At zero concentration, the fluorescence quantum yield $\Phi_0 = k_{\varphi}\tau_0$, and fluorescence lifetime $\tau_0 = 1/(k_{\varphi} + k_n)$. Taking into account that the excimer dissociation probability $\gamma = k_{\text{de}}/(k_{\varphi\text{e}} + k_{\text{ne}} + k_{\text{de}})$ we get:

$$\frac{\Phi_0}{\Phi} = \left\{1 + \tau_0(1 - \gamma) \sum_{i=1}^{\infty} k_{\text{e}}^i [\text{M}_i]\right\} \frac{C_{\text{m}} + \beta}{C_{\text{m}} + \beta\gamma}. \quad (4)$$

The right-hand side of eq. (4) splits into two terms: the static

$$S = (C_{\text{m}} + \beta)/(C_{\text{m}} + \gamma\beta) \quad (5)$$

and the dynamic one

$$D = 1 + \tau_0(1 - \gamma) \sum_{i=1}^{\infty} k_{\text{e}}^i [\text{M}_i]. \quad (6)$$

The reciprocal of the former expresses the probability that absorption of a quantum leads to the formation of an excited molecule M^*. The dynamic term, analogous to the Stern–Volmer equation, describes the diffusion-dependent deactivation of M^* molecules.

As will be shown in the discussion, k_{e}^i values do not differ much from one another, and, in most cases, all diffusional quenching processes can be described by the common rate constant k_{e}. Denoting the colligative concentration $C' = \Sigma_{i=1}^{\infty} [\text{M}_i]$ and quenching constant $\kappa = k_{\text{e}}\tau_0$ we obtain finally:

$$\frac{\Phi_0}{\Phi} = [1 + \kappa C'(1 - \gamma)] \frac{C_{\text{m}} + \beta}{C_{\text{m}} + \gamma\beta}. \quad (7)$$

C_{m} and C' are determined by the concentration C and the association constants $K_n = [\text{M}_n]/C_{\text{m}}[M_{n-1}]$. So, eq. (7) expresses the fluorescence quantum yield Φ as a function of C and of the constant parameters K_n, κ, γ, α_n and Φ_0. The hypochromicity coefficients α_n may be found from absorption measurements. In principle, all other parameters can be calculated by iteration if the values of the function $\Phi = f(C)$ are known. However, the number of the equilibrium constants which can be determined is limited in practice, and depends on the solubility of the investigated compound, its stacking ability and accuracy of the fluorescence quantum yield measurements.

The main problem of the calculations lies in separation of the parameters. At low concentrations, function (7) is linear (see fig. 1) with a slope:

$$C_{\text{m}} \xrightarrow{\lim} 0 \quad \text{d}(\Phi_0/\Phi)/\text{d}\, C_{\text{m}} = \kappa(1 - \gamma) + 2k_2\alpha_2(1 - \gamma). \quad (8)$$

This value can, thus, be determined quite accurately. Nevertheless, separate determination of parameters κ, K_2 and γ may be difficult. The fraction of stacked molecules increases with concentration. The consequent increase of the static term S is compensated, however, by a decrease of the dynamic term D owing to a drop of the colligative concentration C'. Thus, only a small deviation of function (7) from linearity may be observed. In such a case, the separation of parameters is practically impossible on the basis of fluorescence quantum yield measurements alone. This difficulty can be overcome by using a quencher method. In the presence of a quencher (Br^- or I^- anions, for example) the fluorescence lifetime is shortened, and κ constant decreases proportionally. If the stacking equilibrium remains undisturbed, the only effect of the quencher will be a change of the dynamic term. Comparison of $\Phi = f(C)$ functions measured for solutions with and without the quencher leads to the separation of the parameters.

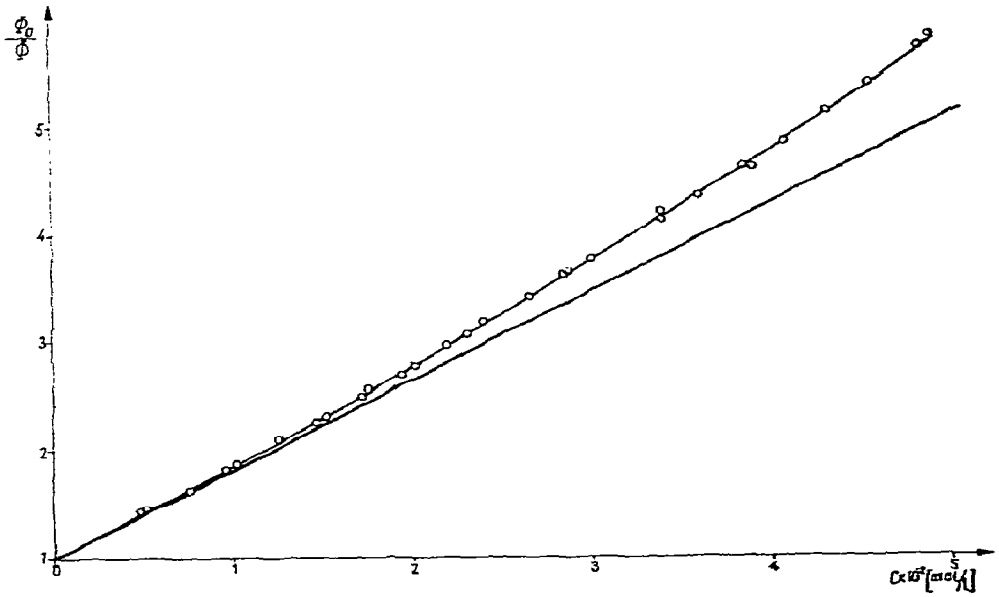

Fig. 1. Reciprocal of the relative fluorescence quantum yield of 2-AP as a function of concentration at 39.6°C. The interpolation curve was calculated (see text) from eq. (7). The slope of the straight line is given by eq. (8).

3. Experimental

3.1. Materials and methods

2-Aminopurine (2-AP) was a Sigma Chemical Co. product. The material was chromatographically homogeneous and was used without purification.

The emission and absorption spectra of 2-AP aqueous solutions do not depend on concentration and temperature. The relative fluorescence quantum yields of the samples were hence determined from the intensity of the fluorescence signals measured at the apparent maximum (370 nm) with excitation at the absorption maximum (305 nm). The apparatus and method of measurements have been described previously [2].

The investigated solutions were not buffered. Their pH varied between 6.5 and 7.0. In this range of pH the fluorescence quantum yield of 2-AP (pK_a = 3.5) remains constant [15].

Absorption spectra were measured with a Cary 118 spectrometer. For absorption measurements at 305 nm, an interference filter was placed behind the samples to cut off their fluorescence light.

2-AP fluorescence lifetimes were measured either on a single photon-counting fluorimeter in the Centre de Biophysique Moléculaire, CNRS, Orléans, or by means by a phase fluorometer in the Institute of Physics, Polish Academy of Sciences, Warsaw. The latter method is more accurate in the case of short lifetimes, below 2 ns.

3.2. Calculations

Calculations were made on an Odra 1204 computer using a Mincon-20 programme (20) in a version without derivatives. The programme finds iteratively the values of parameters X_i for which the function $F(X_i)$, calculated by a procedure given by a user, reaches its minimum. In all procedures used by us, the $F(X_i)$ function was the sum of the square relative deviations of the experimental points from the corresponding interpolation curve.

Mathematical analysis shows that there is no more than one minimum of the function $F(\Phi_0, \kappa, K_n, \gamma)$ if the interpolation curve is given by eq. (7) [21]. So, there is no more then one set of the parameters Φ_0, κ,

K_n and γ which do describe properly the observed changes of the fluorescence quantum yield with concentration. Furthermore, the result of calculation of Φ_0, κ, K_2 and γ depends but slightly on a proposed model of association provided that the model permits to obtain a good fit of function (7) to the experimental data. This latter remark is a consequence of the general validity of eq. (8) which was derived without any assumptions concerning the association model.

Accuracy, with which the parameters can be determined, depends on a broadness of the function F minimum and consequently on accuracy of the fluorescence quantum yield measurements and possible coupling between the parameters (see comments on eq. (8)).

In order to reduce the number of parameters describing the stacking equilibrium, it was assumed that all association constants K_n, except the dimerisation one, K_2, are equal. Concentration and colligative concentration of a solution can be described as follows [22]:

$$C = \frac{\psi C_m}{(1 - KC_m)^2} - \psi C_m + C_m, \tag{9}$$

$$C' = \frac{\psi C_m}{1 - KC_m} - \psi C_m + C_m, \tag{10}$$

where K is the polymerisation constant and $\psi = K_2/K$ the cooperativity parameter.

3.3. Choice of the quencher

Potassium bromide is an effective quencher of 2-AP fluorescence. The Stern–Volmer law is valid up to a 1M concentration of KBr in diluted (10^{-4} mol/ℓ) as well as in concentrated (1.5×10^{-2} mol/ℓ) solutions of 2-AP at 20°C. It proves that an association between Br^- anions and 2-AP molecules does not occur in the ground state, and that KBr does not alter the stacking equilibrium of 2-AP. These conclusions are in agreement with the communications published so far [23,24]. Changes of fluorescence lifetime brought about by the quencher were proportional within accuracy of measurements, to the drop of the fluorescence quantum yield. For detailed results and discussion of 2-AP fluorescence quenching by bromide anions see [21].

Table 1
Extinction coefficients of 2-AP aqueous solution as a function of concentration at 20°C

C (10^{-2} mol/ℓ$^{-1}$)	ϵ^{exp} (ℓ/mol^{-1} cm^{-1})	ϵ^{int} (ℓ/mol^{-1} cm^{-1})	$\Delta\epsilon^{exp}$ (%)
0.350	6.006	6.051	−0.8
0.474	6.082	6.049	0.5
0.917	6.077	6.042	0.6
1.399	6.050	6.034	0.3
1.436	6.030	6.033	−0.1
1.821	6.077	6.028	0.8
1.886	6.014	6.027	−0.2
2.173	6.053	6.023	0.5
2.354	5.995	6.020	−0.4
2.546	5.993	6.017	−0.4
2.695	5.947	6.015	−1.1
2.923	5.990	6.012	−0.4
3.299	5.998	6.007	−0.2
3.315	6.011	6.008	0.1
3.674	6.014	6.004	0.2
3.796	5.992	6.002	−0.2
4.270	5.943	5.996	−0.9
4.753	6.090	5.991	1.6

ϵ^{exp} experimental; ϵ^{int} calculated from eq. 11 for α =0.97.

3.4. Results and discussion

a) Hypochromicity coefficients α_n. Extinction coefficients of 2-AP aqueous solutions of various concentrations measured at 20°C at absorption maximum 305 nm are presented in table 1, column 2. A small hypochromic effect of 2-AP association corresponds with the previous observations of relatively low hypochromicity in 2-AP polynucleotides [16]. Only a very small drop (ca. 1%) of extinction is observed in the most concentrated solutions. Therefore α_n values are close to unity, and no experimental distinction between them is possible. By using a common hypochromicity coefficient $\alpha = \epsilon_n/\epsilon$ for all aggregates, the observed extinction coefficients can be expressed as follows:

$$\epsilon_{obs} = \epsilon(1 - \alpha)C_m/C + \epsilon\alpha \tag{11}$$

and β factor (eq. (7)):

$$\beta = \alpha(C - C_m). \tag{12}$$

Assuming $\alpha = 1$, we calculated the stacking parameters K and ψ as described below. Then the C_m values were found, and $\alpha = 0.97$ was calculated by fitting function (11) to the experimental points. This value was used in further calculations. The values of the extinction coefficients interpolated by eq. (11), along with the deviations of the experimental points from the interpolation curve, are given in columns 3 and 4 of table 1.

b) Calculation of parameters κ, γ, K and ψ.

Two series of 2-AP solutions, which covered the range of concentrations 10^{-4} to 5×10^{-2} mol/ℓ were prepared. One of them consisted of 15 solutions without KBr. The other 22 solutions contained KBr in a constant concentration of 0.15 mol/ℓ. Relative quantum yield of each solution was measured at ten temperatures within the range 0.6 to 88.4°C. For each temperature independently, the parameters Φ_0, κ, γ, K and ψ were calculated by fitting function (7) to the experimental points simultaneously for both series of solutions. It was assumed that the ratio of parameters Φ_0/κ as well as the association constants and γ parameter were not altered by the quencher (see choice of quencher).

At all temperatures perfect fits were obtained. Only statistical dispersion of the experimental points was observed. The mean square deviation of the measured Φ values from the interpolation curves was about 1% and in the worst case, at 0.6°C, it reached 1.3%.

The Φ_0 values calculated for the solutions without KBr were interpolated with the use of the equation found previously [2]:

$$\Phi_0 = 0.9/\{1 + 2932 \exp(-6.243 \text{ kcal/mol}/RT)\}, \quad (13)$$

Then, taking Φ_0 as invariable, the calculations of the other parameters were repeated.

For all temperatures $\gamma = 0$ was obtained. The calculated values of κ are given in table 2, column 2. The fluorescence lifetime of 12 ns was measured in a diluted 2-AP aqueous solution at 20°C by the single-photon counting technique. By means of eq. (13) the lifetime τ_0 at other temperatures could be calculated, and consequently the excimer formation rate constants, $k_e = \kappa/\tau_0$, given in column 3 of table 2.

According to Noyes' treatment [25], the k_e constant can be expressed as the following function of temperature:

$$k_e = k_{dif}\left\{1 + \frac{k_{dif}}{\varphi\rho^2\sqrt{8\pi kT/\mu}\exp(-\Delta E/RT)}\right\}^{-1} \quad (14)$$

Table 2
2-AP fluorescence quenching constant κ and excimer formation rate constants k_e

Temp. (°C)	κ^{exp} (ℓ mol^{-1})	k_e^{exp} (10^9) (mol^{-1} s^{-1})	k_e^{int} (10^9) (mol^{-1} s^{-1})	Δk_e^{exp} (%)	κ^{int} (ℓ mol^{-1})
0.6	32.66	2.60	2.62	−0.5	32.83
10.4	43.45	3.51	3.49	0.7	43.15
20.1	53.82	4.43	4.45	−0.4	54.03
29.9	65.03	5.49	5.51	−0.3	65.25
39.6	76.52	6.67	6.65	0.3	76.28
49.4	87.34	7.92	7.88	0.5	86.94
59.1	97.14	9.23	9.19	0.5	96.65
68.9	105.70	10.63	10.58	0.4	105.24
78.6	112.12	12.03	12.05	−0.2	112.30
88.4	116.58	13.46	13.60	−1.0	117.78

exp - experimental; int - calculated from eq. (14) for $\varphi = 1$, $\Delta E = 2$ kcal/mol and $\rho = 8.3$ Å.

The diffusion rate constant k_{dif} is given by the Smoluchowski-Einstein equation:

$$k_{dif} = \frac{8RT}{3000\eta}\xi, \quad \text{where } \xi = \tfrac{1}{2}(\rho_A + \rho_B)\cdot(1/r_A + 1/r_B), \quad (15)$$

φ is the steric factor, μ reduced mass of the reacting molecules, ΔE activation energy, η viscosity of solution, ρ_A, ρ_B, r_A and r_B the diffusion and reaction radii of the reacting molecules A and B, respectively, $\rho = \rho_A + \rho_B$.

The best fit of function (15) to the experimental k_e values was obtained for $\xi = 1$, $\Delta E = 2$ kcal/mol and the product $\sqrt{\varphi}\rho = 8.3$ Å. Taking into account that the sum of Van der Waals radii of two 2-AP molecules is about 6 Å, the most probable values of the steric factor and the encounter distance are $\varphi = 1$ and $\rho = 8.3$ Å. The constants k_e^{int} interpolated in this way are given in column 4 of table 2. The next column shows the relative differences between k_e and k_e^{int} values.

The erros of ρ and ΔE, due to dispersion of the experimental points, do not exceed ±1 Å and ±0.1 kcal/mol, respectively. Nevertheless, they may be much higher than that because of possible k_{dif} errors. For many compounds, the actual diffusion rate constants differ significantly from those calculated by eq. (15). We tried to vary the k_{dif} values multiplying them by

several arbitrary factors, and subsequently recalculated ρ and ΔE. The best fit of function (14) was obtained for diffusion rate constants calculated directly from eq. (15), but even large deviations from it cannot be excluded. In any case, ΔE is not lower than 1.5 and not higher than 3 kcal/mol and ρ lies between 7 and 10 Å.

Let us consider the errors of the κ values, due to the simplification introduced by the assumption that all k_e^i constants are the same for all M_i aggregates. We estimated parameter ξ for dimers, trimers and tetramers, assuming that the diffusion and reaction radii equalled one another and were larger by 2.3 Å than the Van der Waals ones. For $\Delta E = 2$ kcal/mol, at 0.6°C, the k_e^i values are: $k_e^2 = 1.01\ k_e^1$, $k_e^3 = 1.04\ k_e^1$ and $k_e^4 = 1.06\ k_e^1$. Fractions of colligative concentration corresponding to monomers, dimers, trimers and higher aggregates are 0.74, 0.17, 0.06 and 0.03, respectively, in the most concentrated ($4.5\ 10^{-2}$ mol/ℓ) of the investigated solutions, at 0.6°C. From these data, the difference was calculated between constant κ for the reaction with monomers, and κ in the true solution. It equals 0.6% and remains within the error limits of the measurements. At higher temperatures and in less concentrated solutions this difference becomes close to zero.

Pörschke and Eggers [22] found an activation energy of about 6 kcal/mol for association of N^6, N^9-dimethyladenine in the ground state. This energy is supposed by them to be necessary for removing the last solvation layer of water from between the associating molecules. Our results seem to support this suggestion. In the first, diffusion-controlled step of excimer formation a transient complex of two molecules separated by a solvation layer is formed. So, the encounter distance is equal to the sum of two molecular radii puls the solvation layer thickness. In the second step of the reaction, controlled by the activation energy of a few kcal/mol, the water molecules are removed.

The mechanism of association in the ground state is, most probably, the same. Only a very small change in the activation energy, due to a difference between the solvation energies of the excited and unexcited molecules, is to be expected. Hence, the k_e values presented here can be considered as 2-AP stacking rate constants.

In the last column of table 2 the κ^{int} constants, calculated from k_e^{int} values are presented. They were used in further calculations.

Finally, the stacking constants K and ψ were determined taking all other parameters found previously as established. The calculations were based only on the results obtained for solutions containing KBr. In the presence of KBr, the dynamic factor D of fluorescence quenching is about four times lower, and the stacking parameters can be calculated more accurately being less perturbed by k_e errors.

The calculated K_2 values are presented in table 3, column 2. They can be interpolated quite well by the Van't Hoff relationship. The enthalpy and entropy of dimerization are: $\Delta H_2 = -4.17$ kcal/mol, $\Delta S_2 = -10.9$ e.u. The interpolation line $\ln K_2 = f(1/T)$ is shown in fig. 2 together with the experimental points.

Table 3
2-AP dimerization (K_2) and polymerization (K) constants

Temp. (°C)	K_2^{exp} (ℓ mol^{-1})	$^{+}K_2^{int}$ (ℓ mol^{-1})	$^{+}\Delta K_2^{exp}$ (%)	$^{++}K_2^{int}$ (ℓ mol^{-1})	$^{++}\Delta K_2^{exp}$ (%)	K^{exp} (ℓ mol^{-1})	$^{+++}K^{int}$ (ℓ mol^{-1})	$^{+++}\Delta K^{exp}$ (%)
0.6	9.59	8.88	8.0	9.38	2.2	14.09	14.10	−0.1
10.4	6.68	6.81	−1.9	6.91	−3.2	12.80	12.65	1.2
20.1	5.41	5.33	1.4	5.26	2.9	11.62	11.75	−1.1
20.9	3.96	4.23	−6.5	4.10	−3.5	11.37	11.23	1.2
39.6	3.30	3.41	−3.2	3.29	0.5	10.65	11.04	−3.5
49.4	2.66	2.78	−4.5	2.69	−1.1	11.27	11.12	1.3
59.1	2.28	2.30	−1.2	2.25	1.2	11.67	11.46	1.8
68.9	1.98	1.92	3.3	1.91	3.7	11.89	12.05	−1.4
78.6	1.62	1.62	0.0	1.66	−2.3	13.14	12.91	1.7
88.4	1.45	1.38	5.4	1.46	−0.4	13.93	14.09	−1.1

exp - experimental; int - calculated from the equations: (+) $\Delta G_2 = 10.9\ T - 4170$, (++) $\Delta G_2 = -0.0308\ T^2 + 30.3\ T - 7213$, (+++) $\Delta G_2 = -0.0797\ T^2 + 45.4\ T - 7893$.

In columns 3 and 4 of table 3 the interpolated values of K_2^{int} and the relative deviations of the experimental K_2 values from the interpolated ones are given. The deviations are not only of statistical character. A better interpolation can be achieved by assuming that the dimerization free enthalpy is described by the following second-order polynomial:

$$\Delta G_2 = -0.0308\ T^2 + 30.3\ T - 7213. \qquad (16)$$

The values K_2^{int}, interpolated in this way, are given in column 5 of table 3 and deviations of the experimental K_2 values from the interpolation curve in column 6 of this table.

The calculated polymerization constants K are presented in table 3, column 7. Changes of K with temperature can be described correctly only by the second-order relationship:

$$\Delta G = -0.07971\ T^2 + 45.396\ T - 7893.5. \qquad (17)$$

The values K^{int}, interpolated by using eq. (17), and the relative deviations of the experimental K values from the interpolated ones are given in columns 8 and 9 of table 3. The function $\ln K = f(1/T)$, calculated from eq. (17), is shown in fig. 2. The function reaches its minimum at about 40°C. Above this temperature the enthalpy of polymerization, ΔH, is positive. At 20°C $\Delta H = -1.04$ kcal/mol and $\Delta S = 1.34$ e.u.

The 2-AP dimerization constants, as well as dimerization enthalpy and entropy, are typical for purine bases [1]. The polymerization constants are higher than the dimerization ones. It should be stressed here that the isodesmic model ($\psi = 1$) does not described adequately the observed 2-AP fluorescence quenching. For this model, systematic deviations of function (7) from the experimental points were observed, especially at low temperature.

This result agrees with some communications published so far. The data of Mukerjee and Gosh for methylene blue [26] and those of Magar and Steiner for inosine, cytidine and uridine [27] indicate that the association of those compounds is also a cooperative process, and that the K_2/K ratio changes with temperature approximately in the same way as in the case of 2-AP.

Relatively high enthalpy and entropy of polymerization seem to indicate, as suggested by Mukerjee and Gosh [26], that the hydrophobic effect plays a major role in stabilization of polymers. The observed changes

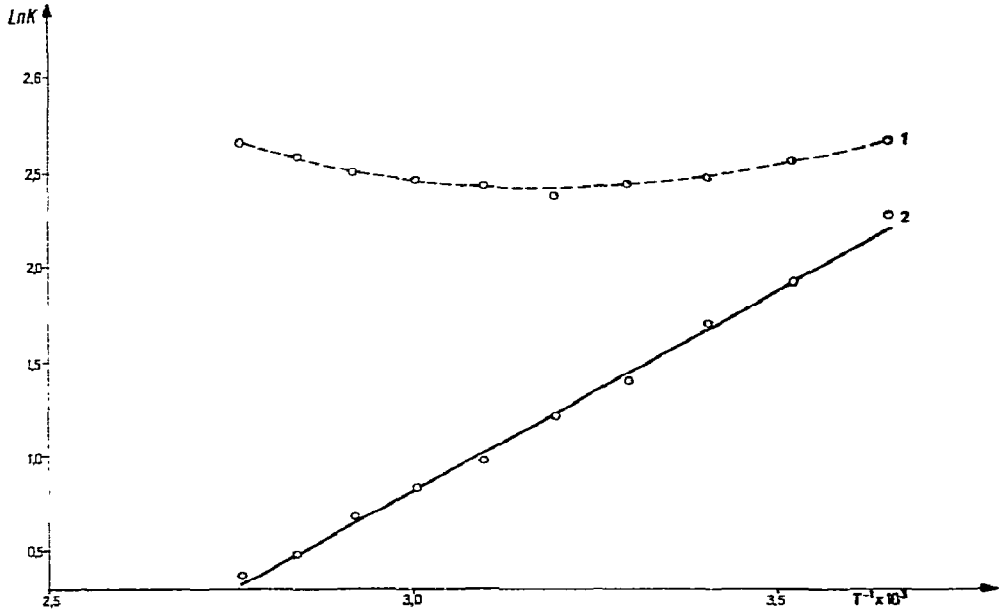

Fig. 2. Van't Hoff plots of 2-AP polymerization (1) and dimerization (2) constants. The interpolation curves were calculated for $\Delta H_2 = -4.17$ kcal/mol, $\Delta S_2 = -10.9$ e.u. and $\Delta G = -0.0797\ T^2 + 45.4\ T - 7893$, respectively.

of the thermodynamical parameters with temperature may be attributed to the competition between hydrophobic and electrostatic forces which contribute to stabilization of aggregates. The problem has recently been discussed by Plesiewicz et al. [28]. At low temperature, electrostatic interactions between the stacked molecules are particularly pronounced because of their negative enthalpy, and determine, to a high extent, the relative orientation of the associated molecules. With increase of temperature, the hydrophobic effect, characterized by small positive enthalpy and large positive entropy, becomes a dominant factor. Consequently, a rearrangement of the stacked molecules occurs, and the contribution of the hydrophobic interactions to the free enthalpy of the association increases. Accordingly, an increase of both entropy and enthalpy of polymerization is observed. In the case of dimerization the same phenomenon seems to occur (see eq. (16)), although it is only little noticeable as the hydrophobic effect plays a less important role in dimer stabilization.

Unfortunately, our results do not permit conclusions as to whether the equilibrium constants of trimer and higher polymers formation are actually equal to one another. If it is not the case, the calculated K parameters are linear functions of several different equilibrium constants, each of them describing a particular step of the association. The distribution of the stacked molecules among the various aggregates in a concentrated (4.5×10^{-2} mol/ℓ) 2-AP solution is shown in fig. 3. It corresponds to the contribution of the various aggregates to static fluorescence quenching. If the enthalpy and entropy of the association increase systematically with the size of the aggregate, the observed temperature dependence of the thermodynamical parameters of polymerization may be partly due, especially above 60°C, to the increasing fraction of highly aggregated molecules.

4. Conclusions

The fluorescence quenching method proved to be more sensitive and accurate than any other used so far

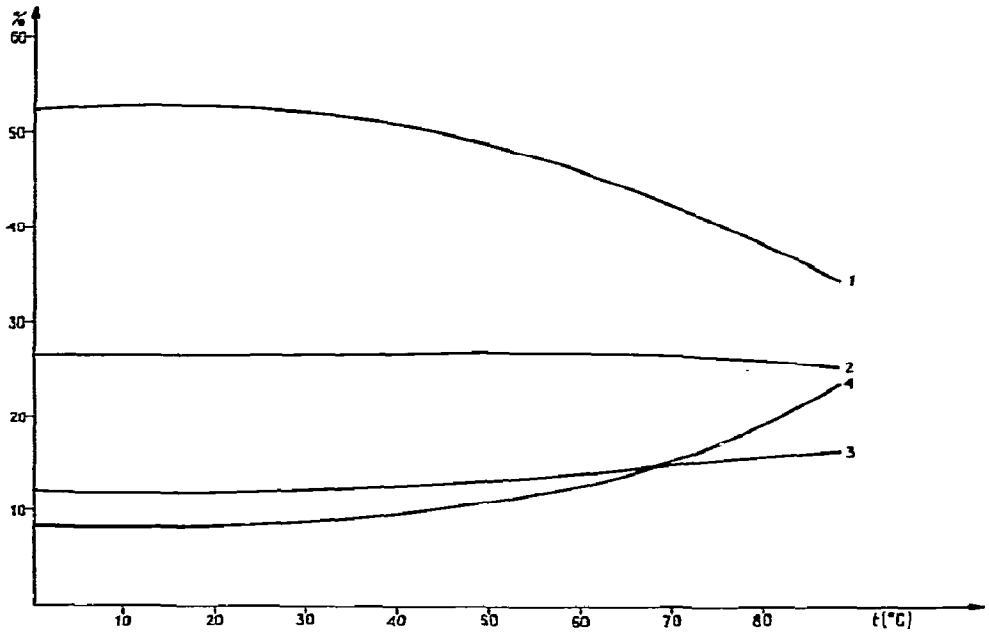

Fig. 3. Fractions of 2-AP molecules stacked in 1) dimers, 2) trimers, 3) tetramers and 4) higher aggregates as a function of temperature at total 2-AP concentration of 4.5×10^{-2} mol/l.

ii. stacking autoassociation dynamics and equilibrium investigations. Its accuracy is due to the fact that not only the colligative concentration of a solution (dynamic term), but also the fraction of unstacked molecules (static term) are directly and independently calculated from the fluorescence lifetime and quantum yield measurements. Besides, the measured signal depends largely on association. Actually, if $\gamma = 0$, the fluorescence of stacked molecules is fully quenched. The advantages of the method become obvious when taking into account that the stacking equilibrium constant of 2-AP can be determined by vapour-pressure osmometry merely with an error of about 50% because of the low solubility of the compound. At the available concentrations, it is impossible to measure the 2-AP stacking rate constants by any method used so far.

The accuracy of the fluorescence quenching method makes possible rejection of rather rough simplifications of the isodesmic model and separate determination of the equilibrium constant of at least one single step of the association, namely the dimerization constant.

The limitations of the method will be discussed in the third part of this work [11].

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