Biophysical Chemistry 6 (1977) 223–229

INVESTIGATIONS ON PURINE AND PYRIMIDINE BASES STACKING ASSOCIATIONS IN AQUEOUS SOLUTIONS BY THE FLUORESCENCE QUENCHING METHOD. II. HETEROASSOCIATION BETWEEN 2-AMINOPURINE AND THYMIDINE

Andrzej BIERZYŃSKI, Hanna KOZŁOWSKA and K.L. WIERZCHOWSKI
Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Rakowiecka 36, 02–532 Warszawa, Poland

Received 4 August 1976
Revised manuscript received 29 October 1976

Heteroassociation between A and B compounds in liquid solution was considered. Provided that concentration of A molecules is low, a general equation describing fluorescence quantum yield and lifetime of compound A as a function of B molecules concentration was derived. The heteroassociation between 2-aminopurine and thymidine in aqueous solutions was examined within the range of temperatures 0 to 90°C. The equilibrium constants of the first step of association, namely heterodimer formation, were determined and its thermodynamic parameters ($\Delta H = -2.76$ kcal/mol, $\Delta S \approx -5.9$ e.u.) were calculated. The observed changes of the stacking rate constants with temperature confirm the two-step mechanism of the reaction. The activation energy (~2.7 kcal/mol) and the encounter distance (~10.7 Å) are only slightly larger than in the case of 2-aminopurine autoassociation, most probably because of a stronger solvation of thymidine molecules.

1. Introduction

The theoretical basis and practical application of the fluorescence quenching method to investigation of stacking autoassociations have been discussed in the first part of this paper [1]. Here, the application of the method to investigation of equilibrium and kinetics of stacking associations between molecules of two different compounds is described. The results obtained for the 2-aminopurine + thymidine aqueous system illustrate the practical problems to be envisaged in such investigations.

2. Theoretical

In a two-component solution of A and B compounds both auto- and heteroassociations are possible. When C_A, the concentration of compound A, is maintained low enough, the autoassociation of A molecules can be neglected, and the concentration of heteroaggregates with more than one A molecule is practically zero. Besides, the concentration of heteroaggregates AB_i, composed of one molecule A and $i \geq 1$ molecules B, is low as compared with C_B, so that the autoassociation of B molecules is not disturbed by the presence of compound A.

The excitation of a stacked molecule A leads to exciplex formation. The exciplexes can be formed, as well, by the reaction of excited molecules A with molecules B or their aggregates. Our aim was to describe the fluorescence quantum yield of compound A as a function of compound B concentration, C_B.

The following assumptions, thoroughly discussed in the first part of this paper [1] are made:

1) After excitation of a stacked molecule A, the probability of exciplex formation is equal to 1.
2) For all kinds of exciplexes, realized in the system, the probability of dissociation $\gamma = k_{dex}\,\tau_{ex}$ is the same. k_{dex} and τ_{ex} are the exciplex dissociation rate constant and the exciplex lifetime.
3) All exciplex formation processes

$$A^* + B_i \rightarrow Ex, \qquad (I)$$

independently of the size of aggregate B_i occur with the same rate constant k_{ex}.
4) Resonance transfer of excitation energy can be neglected.

Let Φ_0 denote the fluorescence quantum yield of the solution of compound A at C_A concentration.

After addition of component B the fluorescence quantum yield drops to the value Φ. The similarity of the quenching mechanisms in hetero- and autoassociating systems suggests that in both cases the general form of quenching functions should be the same:

$$\Phi_0/\Phi = DS. \tag{1}$$

The reciprocal of the static term S is the quantum yield of molecules A excitation. In other words, it is a fraction of the excitation light absorbed effectively by a solution. Extinction of the solution is equal to:

$$\epsilon_{ex} = \epsilon_{Bm}(C_{Bm} + \beta_B) + \epsilon_{Am}(C_{Am} + \beta_A), \tag{2}$$

where ϵ_{Am}, ϵ_{Bm}, C_{Am}, and C_{Bm} denote the extinction coefficients, at the excitation wavelength, and concentrations of monomers A and B.

$$\beta_A = \sum_{i=1}^{\infty} \frac{\epsilon_{ABi}}{\epsilon_{Am}} [AB_i], \qquad \beta_B = \sum_{n=2}^{\infty} i \frac{\epsilon_{Bi}}{\epsilon_{Bm}} [B_i],$$

ϵ_{ABi} is the extinction coefficient of heteroaggregate AB_i and ϵ_{Bi} the extinction coefficient of molecules B associated in aggregates B_i. The coefficients $\epsilon_{ABi}/\epsilon_{Am} = \alpha_{Ai}$ and $\epsilon_{Bi}/\epsilon_{Bm} = \alpha_{Bi}$ describe the hypochromicity due to stacking hetero- and autoassociations.

The static term can thus be expressed as follows:

$$S = \frac{(\epsilon_{Bm}/\epsilon_{Am})(C_{Bm} + \beta_B) + C_{Am} + \beta_A}{C_{Am} + \beta_A \gamma_{ex}}. \tag{3}$$

At a very low C_A concentration, when all other dynamical quenching processes, apart from reaction (I), are negligible, the dynamic term D is:

$$D = 1 + \tau_0 k_{ex}(1 - \gamma_{ex}) \sum_{i=1}^{\infty} [B_i], \tag{4}$$

where τ_0 is the natural fluorescence lifetime of molecules A.

The fluorescence measurements at such a low concentration are difficult in practice. Two other ways of quenching:

$$A^* + A \longrightarrow E, \tag{II}$$

and

$$A^* + AB_i \longrightarrow Ex, \tag{III}$$

must then be taken into account and the necessary correction introduced into eq. (4):

$$D = 1 + \tau' k_{ex}(1 - \gamma_{ex}) \sum_{i=1}^{\infty} [B_i], \tag{5}$$

$$\tau' = \tau_0/[1 + \tau_0(1 - \gamma) k_e C_{Am} + \tau_0(1 - \gamma_{ex}) \sum_{i=1}^{\infty} [AB_i] k_{ex}^i] \tag{6}$$

where γ is the probability of excimer E dissociation, k_e and k_{ex}^i are rate constants of the reactions (II) and (III), respectively.

Because of a low C_A concentration, the difference between τ_0 and τ' is small, and even a rough simplification of eq. (6) is possible. Putting $\gamma = \gamma_{ex}$ and $k_e = k_{ex}^i$ one obtains:

$$\tau' = \tau_0/[1 + \tau_0 k_e(1 - \gamma) C_A], \tag{7}$$

τ' is then the fluorescence lifetime of compound A in the solution of C_A concentration.

Introduction of eqs. (3) and (5) into eq. (1) gives finally:

$$\Phi_0/\Phi = \left\{1 + \kappa'(1 - \gamma_{ex}) \sum_{i=1}^{\infty} [B_i]\right\} \times \frac{(\epsilon_{Bm}/\epsilon_{Am})(C_{Bm} + \beta_B) + C_{Am} + \beta_A}{C_{Am} + \beta_A \gamma_{ex}}, \tag{8}$$

where $\kappa' = \tau' k_{ex}$.

The same expression was obtained in a rigorous way, when comparing the rates of all processes leading to formation and decay of the excited species in a steady-state equilibrium system. We describe the autoassociation equilibrium using the model applied previously [1]. For all steps of the association the equilibrium constants $K_B = [B_{n+1}]/C_{Bm}[B_n]$ are supposed to be equal to one another apart from the dimerization constant: $K_{dB} = [B_2]/C_{Bm}^2$. The concentration C_B and colligative concentration C_B' can be expressed by the equations:

$$C_B = \frac{\psi_B C_{Bm}}{(1 + K_B C_{Bm})^2} - \psi_B C_{Bm} + C_{Bm}, \tag{9}$$

$$C_B' = \frac{\psi_B C_{Bm}}{1 + K_B C_{Bm}} - \psi_B C_{Bm} + C_{Bm}, \tag{10}$$

where $\psi_B = K_{dB}/K_B$. Similarly, we describe the heterodimer, AB, formation by the equilibrium constant $K_{dAB} = [AB]/C_{Am} C_{Bm}$, and all other heteroassocia-

tion processes by the same constant $K_{AB} = [AB_n]/[B_n]\, C_{Am}$. Hence:

$$C_{Am} = \frac{C_A}{1 + K_{AB}(C'_B - C_{Bm} + \psi_{AB} C_{Bm})}, \qquad (11)$$

where $\psi_{AB} = K_{dAB}/K_{AB}$. According to our basic assumptions eqs. (9) and (10) are valid not only in pure solution of B but also in that containing compound A. In the latter case, however, the colligative concentration is not only a sum of B_n aggregates concentration, but

$$C' = \sum_{i=1}^{\infty} [B_i] + \sum_{i=1}^{\infty} [AB_i]. \qquad (12)$$

Thus, eq. (8) takes the form:

$$\Phi_0/\Phi = [1 + \kappa'(1 - \gamma_{ex})(C'_B + C_{Am} - C_A)] \times \frac{(\epsilon_{Bm}/\epsilon_{Am})(C_{Bm} + \beta_B) + C_{Am} + \beta_A}{C_{Am} + \beta_A \gamma_{ex}}. \qquad (13)$$

Because of the simplifications made in the derivation of eq. (13), its applicability is limited to low concentrations of compound A. Namely, the following values:

$$\frac{[AB]}{C_{Bm}} = K_{dAB} C_{Am}, \quad \frac{[AB_i]}{[B_i]} = K_{AB} C_{Am},$$

$$\frac{[AA]}{C_{Am}} = K_{dA} C_{Am},$$

(where K_{dA} is the dimerization constant of compound A), should be small; smaller than 1% in practice. For association constants not exceeding 10 ℓ/mol, it means that C_A should be lower than 10^{-3} mol/ℓ. All other limitations of our treatment are given by the assumptions 1–4.

Apart from the extinction and hypochromicity coefficients ϵ_{Am}, ϵ_{Bm}, α_{Ai} and α_{Bi}, which may be found from absorption measurements, the function $\Phi = f(C_B)$, given by eq. (13) along with eqs. (9), (10) and (11), depends on the following parameters: Φ_0, κ', γ_{ex}, K_{dB}, K_B, K_{dAB} and K_{AB}. Only some of them can be calculated iteratively by fitting functions (13) to the experimental data. Mathematical analysis of eq. (13) shows that the value of γ_{ex} cannot be found in this way. As the other are parameters concerned, it can be proved, similarly as in the case of autoassociation [1], that there is no more than one set of their values which describes properly the observed changes of Φ with C_B concentration [2]. Nevertheless, because of a coupling between the parameters K_{dB}, K_B and K_{dAB}, only one of them can be calculated, in practice, and two others must be known. Besides, similarly as in the case of autoassociation, the parameters κ' and K_{dAB} are strongly coupled with each other. It is necessary to separate them by the quencher method described previously (1).

3. Experimental

3.1. Materials and methods

2-Aminopurine (2-AP) and thymidine were Sigma Chemical Co. products and used without purification. Two series of unbuffered aqueous solutions of 2-AP and thymidine were prepared. Each of them consisted of 17 solutions containing a constant concentration of 2-AP, 10^{-3} mol/ℓ, and various thymidine concentrations within the range 0 to 0.26 mol/ℓ. One of the series contained KBr as quencher at constant concentration of 0.15 mol/ℓ.

Fluorescence signals of the solutions were measured at the apparent maximum, 370 nm, with excitation at 320 nm. The apparatus and method of measurements were described elsewhere [3]. The absorption measurements were made with a Cary 118 spectrometer. The calculation method was described previously [1].

3.2. Results and discussion

The lowest energy absorption bands of 2-AP ($\lambda_{max} = 305$ nm) and thymidine ($\lambda_{max} = 267$ nm) are well separated from one another. At excitation wavelength 320 nm, the ratio of the extinction coefficients is $\epsilon_{Bm}/\epsilon_{Am} = 1.3 \times 10^{-4}$. Hence, the optical density of samples changed with thymidine concentration within the range of a few percent only, and was 0.35, approximately.

It is worth mentioning here that a high spectral purity of compound B is not necessary. The term $\epsilon_{Bm}(C_{Bm} + \beta_B)/\epsilon_{Am}$ in eq. (13) is merely a correction for the inner filter effect, and is always calculated correctly if only the effective absorbances of B material is determined precisely enough.

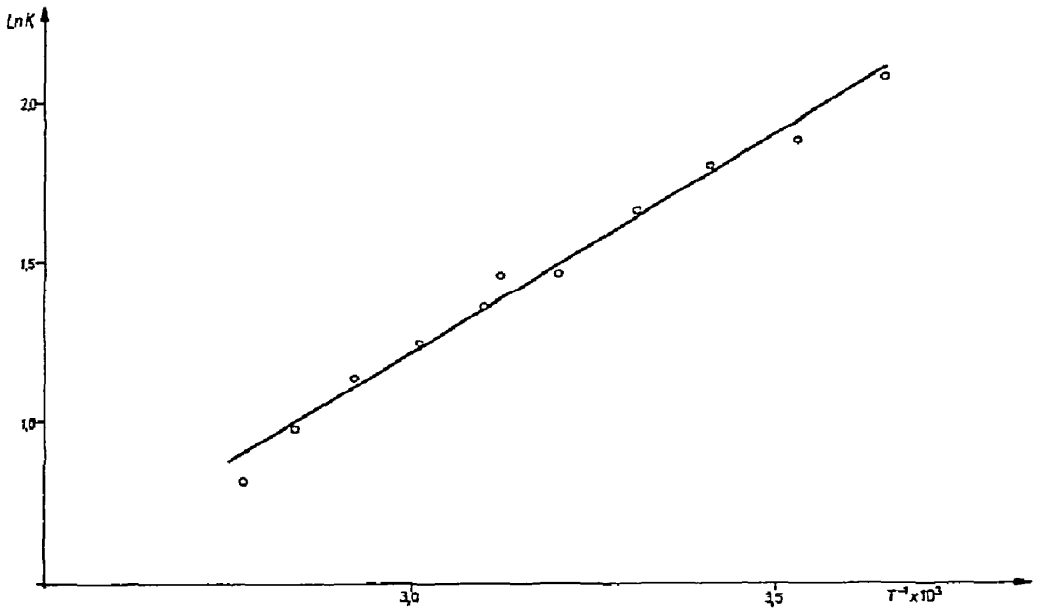

Fig. 1. Van't Hoff plot of the 2-AP + thymidine hetero dimerization equilibrium constant; $\Delta H = -2.76$ kcal/mol, $\Delta S = -5.9$ e.u.

The observed fluorescence signals V are related to the quantum yields of the samples by the equation:

$$V = \text{const.}\ \Phi\,(1 - 10^{-l\epsilon_{ex}}) \tag{14}$$

where l the optical length of the sample, const. the apparatus factor, and ϵ_{ex} is given by eq. (2).

Therefore, eq. (13) can be transformed into a more convenient form:

$$V_0/V = [1 + \kappa'(1-\gamma_{ex})(C'_B + C_{Am} - C_A)]\ S$$

$$\times\ (1-10^{-l\epsilon_{Am}C_A})/(1-10^{-l\epsilon_{ex}}). \tag{15}$$

If the optical densities $l\epsilon_{ex}$ are low, or they do not differ much from $\epsilon_{Am}\ C_A$, eq. (15) can be simplified as follows:

$$V_0/V = [1 + \kappa'(1-\gamma_{ex})(C'_B + C_{Am} - C_A)]$$

$$\times\ C_A/(C_{Am} + \beta_A\,\gamma_{ex}). \tag{16}$$

In such a case, the function V_0/V does not depend on the absorption properties of the compounds if only $\gamma_{ex} = 0$. In our calculations eq. (15) had to be used. However, the shape of the function depends so slightly on the hypochromicity coefficients α_{Ai} and α_{Bi} that their values could be fixed arbitrarily: $\alpha_{Bi} = 1$ and $\alpha_{Ai} = 0.9$.

The fluorescence spectra of all samples were exactly the same, independently of thymidine concentration. It proves that the fluorescence quantum yield of the exciplexes is very low, lower than 5×10^{-4}, and their lifetime probably very short. It could be assumed then that, analogously as in the case of 2-AP exciplexes, $\gamma_{ex} = 0$. This assumption was confirmed by the observed temperature dependence of the dynamic factor discussed below.

Thymidine autoassociation constants were taken from the osmometric data [4] interpreted in terms of the isodesmic model ($\psi_B = 1$). The necessary inter- and extrapolation of K_{dB} values was done assumed a linear relationship $\ln K_{dB} = f(1/T)$ (4).

The fluorescence signals of the samples were measured at ten temperatures within the range 0.6 to 88.4°C. For each temperature independently, the parameters Φ_0, κ', K_{dB} and ψ_{AB} were calculated by fitting function (15) to the experimental points simultaneously for both series of solutions. It was assumed that the ratio of parameters Φ_0/κ' as well as all association constants were not altered by the quencher [1]. For all temperatures a very good fit was obtained. Systematic deviations, observed in a few cases, were not higher than 0.5%. Statistical dispersion was similar as in the case of 2-AP autoassociation (1). The calcu-

Table 1
Stacking equilibrium constants and fluorescence quenching constants in 2-AP + thymidine aqueous solution

t (°C)	K_{dB} (ℓ/mol)	K_{dAB} (ℓ/mol)	ψ_{AB}	κ' (ℓ/mol)
0.6	1.437	8.09	0.38	22.70
10.4	1.208	6.59	0.37	29.21
20.1	1.030	6.09	0.50	35.42
29.9	0.887	5.31	0.61	42.76
39.6	0.773	4.33	0.62	51.10
49.4	0.679	3.94	1.24	57.66
59.1	0.602	3.49	1.48	64.43
68.9	0.538	3.12	11.41	70.33
78.6	0.483	2.66	35.14	75.14
88.4	0.438	2.24	44.17	78.01

K_{dB} thymidine autoassociation constants taken from osmometric data [3]; K_{dAB} 2-AP + thymidine heteroassociation equilibrium constants; $\psi_{AB} = K_{dAB}/K_{AB}$ heteroassociation cooperativity factor; κ' constant of 2-AP fluorescence dynamic quenching by thymidine.

lated values of parameters, along with $K_{dB} = K_B$ constants, are presented in table 1.

The errors of the results are mainly due to the uncertainty of the thymidine autoassociation constants. Unfortunately, the osmometric data are not accurate enough for our purpose. The K_{dB} values calculated from the osmometric measurements strongly depend on the association model on which calculations are based [4]. On the other hand, it is impossible to decide which model is actually correct so, the isodesmic one ($\psi_B = 1$) is adopted. Although the association constants calculated in this way describe very well the measured molality of solutions, they probably fail to express adequately distribution of associating molecules among various kinds of aggregates.

For the temperature of 29.9°C, the iterative calculations of the parameters K_{dB}, ψ_{AB}, K_{dAB} and κ' were made for the cooperative ($\psi_B = 0.5$), isodesmic ($\psi_B = 1$) and anticooperative ($\psi_B = 2$ and 100) models of thymidine association. They were repeated once more under the assumption that $\psi_{AB} = \psi_B = 1$. The results were then compared (see table 2) with those obtained previously for K_B and ψ_B values taken from the osmometric data. In all cases, apart from the latter one where low (0.5%) systematic deviations were observed, a perfect fit of function 15 to the experimental points was obtained. It is obvious hence that the

Table 2
Stacking equilibrium constants and fluorescence quenching constants in 2-AP + thymidine aqueous solution at 29.9°C, calculated for various models of thymidine autoassociation: cooperative ($\psi_B \ll 1$), isodesmic ($\psi_B = 1$) and anticooperative ($\psi_B > 1$)

K_{2B}	ψ_B	ψ_{AB}	K_{dAB}	κ'
0.360	0.5*	5.1	5.16	41.21
0.414	1.0*	8.8	5.17	41.42
0.451	2.0*	8.1	5.16	41.55
0.460	100.0*	250.0	5.15	41.62
0.563	1.0*	1.0*	4.98	42.45
0.887*	1.0*	0.6	5.31	42.76

The values with asterisks were taken as invariable.

thymidine association constants cannot be determined from our measurements. Neither are the ψ_{AB} values certain. Even the order of magnitude of parameter ψ_{AB} is doubtful because of the uncertainty of the thymidine association model. In spite of that, quite exact values of κ' and K_{dAB} can be found. Their errors can be estimated from the data given in table 2 as not exceeding ±3%. Similar estimations made for the other temperatures lead to the same conclusion. In fact, the values of κ' and K_{dB} calculated with assumption of the isodesmic model of auto- and heteroassociations ($\psi_B = \psi_{AB} = 1$) were, for all temperatures, not only close to those listed in table 1, but gave the same values of thermodynamic heteroassociation parameters and practically the same value of stacking activation energy.

The association constants K_{dAB}, given in table 1, can be interpolated fairly well by using Van't Hoff's relationship, $\Delta H = -2.76$ kcal/mol $\Delta S = -5.9$ e.u. The experimental points and the interpolation function are presented in fig. 1. At high (78.6 and 88.4°C) and low (0.6 and 10.4°C) temperatures some small systematic deviations are noted. It is not certain whether they are due to actual temperature changes of heteroassociation enthalpy and entropy, or rather to some systematic errors introduced by the thymidine association constants taken for calculations.

The 2-AP + thymidine association enthalpy is by about 2 kcal/mol higher than that of 2-AP dimerization. This difference is compensated, however, by an increase of entropy. At about 10°C the free enthalpies of both processes are equal and so are the equilibrium

Table 3
Rate constants of 2-AP + thymidine exciplex formation

t (°C)	k_{ex}^{exp} (ℓ/mol × 10^9)	k_{ex}^{int} (ℓ/mol × 10^9)	Δk_{ex} (%)
0.6	1.821	1.820	0.1
10.4	2.382	2.376	0.2
20.1	2.951	2.996	−1.5
29.9	3.661	3.691	−0.8
39.6	4.525	4.450	1.7
49.4	5.325	5.291	0.6
59.1	6.250	6.198	0.8
68.9	7.232	7.190	0.6
78.6	8.254	8.249	0.1
88.4	9.234	9.396	−1.7

exp – experimental; int – calculated from eq. (17) for $\varphi = 1$, $\Delta E = 2.7$ kcal/mol, $\rho = 10.7$ Å.

constants. This result does not agree with the data published so far [5]. According to them, heteroassociation between purine and pyrimidine bases are characterized by lower association constants than the associations between purine bases themselves. This discrepancy is probably due to the fact that our results concern association between single molecules only, whereas the phase partition measurements, on which the previous investigations were based, do not make a distinction between different steps of association. A good agreement of the results can be achieved by assuming that the heteroassociation between 2-AP and thymidine is a strongly anticooperative process: ($\psi_{AB} \gg 1$).

The values of exciplex formation rate constants calculated from the parameters $\kappa' = \tau' k_{ex}$ are given in column 2 of table 3. The lifetimes τ' were calculated from the natural lifetimes τ_0 and selfquenching rate constants of 2-AP measured previously [1]. The 2-AP fluorescence lifetimes depend slightly on the excitation wavelength. Necessary corrections amounting to about 3% were introduced to achieve an agreement with the measured Φ_0 values and KBr quenching constants.

The rate constants k_{ex} were analyzed with the use of the formula:

$$k_{ex} = k_{dif}\left\{1 + \frac{k_{dif}}{\rho^2\varphi\sqrt{8\pi kT/\mu}\exp(-\Delta E/RT)}\right\}^{-1} \quad (17)$$

discussed in the first part of this work [1]. The diffusion rate constants k_{dif} were calculated from the Smoluchowski–Einstein equation:

$$k_{dif} = 8RT/3000\,\eta. \quad (18)$$

The correction for different radii of the reacting molecules (about 3%) was neglected. The following values of the reaction parameters were found: steric factor $\varphi = 1$, encounter distance $\rho = 10.7 \pm 1$ Å, activation energy $\Delta E = 2.7 \pm 0.1$ kcal/mol. As has been mentioned before [1], additional errors of the results $\Delta\rho = \pm 2$ Å and $\Delta E = \pm 1$ kcal/mol are not excluded because of the uncertainty of k_{dif} values. Still, comparison with the results obtained in a similar way for 2-AP excimer formation is possible. The reaction mechanism proposed previously seems to be confirmed. According to expectations, the encounter distance is somewhat larger for the 2-AP + thymidine reaction, because of the larger Van der Waals radius of thymidine as compared with that of the 2-AP molecule. (The estimated difference is about 1 Å.) The increase of activation energy may be attributed, probably, to the higher solvation energy of the thymidine molecule, due to its two hydrophilic cyclicamide groups. Nevertheless, the solvation energy of the reacting molecules seems to be but of secondary importance, since the activation energies of 2-AP excimer formation (2 kcal/mol) and that of 2-AP + thymidine exciplex formation (2.7 kcal/mol) are very similar. This confirms our previous assumption that the rate constants of the reactions between molecules in their ground and excited states are very similar and the k_{ex} values measured by us describe the stacking reaction as well.

The interpolated k_{ex} values and the deviations of the experimental points from the interpolation curve are presented in table 3, columns 3 and 4.

4. Conclusions

Heteroassociations are a particularly difficult object of investigation. It would hardly be possible, by the methods used up till now, to examine the kinetics of the process. All descriptions of heteroassociation equilibrium had to be limited to the isodesmic model only, although its validity is doubtful especially in this case. Association of an A molecule to an aggregate B_n leads to at least two thermodynamically different species with molecule A stacked outside and inside the complex. Thus, it is obvious that not only the

equilibrium constants should be expected to change from one step of the association to another, but even the equilibrium at each of the steps should be described by at least two different association constants. The main advantage of the fluorescence quenching method is its ability of determining separately the stacking equilibrium between A and B monomers. Accordingly, thermodynamical parameters of a single, well defined heteroassociation process can be found.

The shortcomings of the method will be discussed in the third part of this work [6].

Acknowledgement

This investigation was supported by the Polish Academy of Sciences within the Project 09.3.1.

References

[1] A. Bierzyński, H. Kozłowska and K.L. Wierzchowski, Biophys. Chem. 6 (1977) 213.
[2] A. Bierzyński, Ph.D. Thesis, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, (Warsaw, 1977).
[3] A. Bierzyński and J. Jasny, J. Photochem. 3 (1974/75) 431.
[4] E. Plesiewicz, E. Stepień, K. Bolewska and K.L. Wierzchowski, Biophys. Chem. 4 (1976) 131.
[5] P.O.P. Ts'o in: Basic principles of nucleic acids chemistry, ed. P.O.P. Ts'o (Academic Press, New York, 1974) Vol. 1, p. 544.
[6] A. Bierzyński, H. Kozłowska, Z. Proba and K.L. Wierzchowski, Biophys. Chem. 6 (1977) 231.