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FLUORESCENCE QUENCHING AND SPIN-LABEL ELECTRON SPIN RESONANCE STUDIES OF STACKING SELF-ASSOCIATION IN AQUEOUS SOLUTIONS OF 2-AMINOPURINE RIBOSIDE AND ITS 5′-MONO- AND -DIPHOSPHATE

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The autoassociation of 2-aminopurine riboside (rn^{2}Pur) and its 5′-mono- (*P*-rn^{2}Pur) and 5′-diphosphate (*PP*-rn^{2}Pur) in neutral aqueous solutions was investigated using fluorescence quenching and ESR spin-label methods within the range 276 – 358 K. Respective equilibrium constants and thermodynamic functions were derived therefrom assuming two models of infinite autoassociation: (i) an isodesmic one ($K_2 = K_3 = \ldots K_p$), and (ii) one in which $K_2 \neq K_3 = K_4 = \ldots K_p$. Comparative analysis of these data and that of the parent 2-aminopurine, obtained previously, allowed us to formulate the following conclusions: (1) the mechanism of autoassociation of rn^{2}Pur varies with temperature in such a way that at $T = 318$ K the isodesmic model is fulfilled ($K_2 = K_p$); at higher temperatures $K_p/K_2 > 1$, i.e., the process is cooperative, while at lower temperatures it becomes anticooperative ($K_p/K_2 < 1$); (2) at 298 K the tendency to autoassociation decreases in the order: rn^{2}Pur > *P*-rn^{2}Pur > *PP*-rn^{2}Pur; (3) rn^{2}Pur forms highly packed complexes with the bases stacked and the ribofuranose residues interacting via hydrogen bonds or water bridges; (4) autoassociation of *P*-rn^{2}Pur and *PP*-rn^{2}Pur is mainly governed by stacking of the bases, while the ribose phosphate residues attain a *trans* configuration corresponding to the lowest electrostatic repulsion between charged phosphate groups; even at high ionic strength ($I = 0.8$), a positive electrostatic contribution to the free enthalpy of autoassociation is observed; (5) the two methods employed gave similar results for *P*-rn^{2}Pur, but somewhat different ones for rn^{2}Pur because the presence of the spin label (nitroxide stable radical) at the 2′(3′)-OH group of the ribose residue prevents its interaction via hydrogen bonding with an unlabeled one of an adjacent nucleoside.

1. Introduction

Investigations on homo- and heteroassociation of nucleic acid bases in aqueous solutions help in the understanding of the nature of their stacking interactions and allow evaluation of the contribution of these interactions to the thermodynamic stability of ordered polynucleotide structures (for a review, see ref. 1). In the light of equilibrium and thermodynamic data accumulated so far the involvement of both polarization and hydrophobic effects in stacking interactions seems to be well documented. The actual balance of the two effects varies with the structure of interacting bases. Dipole-induced dipole forces are particularly important in interactions of purine derivatives, owing to the high polarizability of their heteroaromatic rings and large bond-dipole moments of polar substituents [2,3]. Substitution with alkyl groups induces around base molecules specific changes in the hydration shells typical of essentially hydrophobic solutes, thus increasing the contribution of hydrophobic interactions to stacking association [4–7]. In fact, hydrophobic interactions were shown to govern predominantly self-association of alkylated uracils and thymines [7], the diketopyrimidine skeleton of which exhibits low polarizability.

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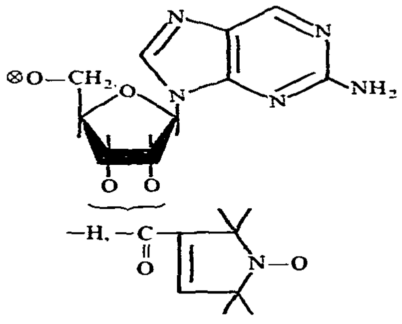
Perhaps the least understood effects remaining are those of substitution of the bases with strongly hydrophilic carbohydrate groups like ribose or deoxyribose and their various phosphate esters, important for the formation of complementary polynucleotide-mononucleotide complexes. Comparative equilibrium and thermodynamic data are available only for a limited number of related base-nucleoside pairs [1,7,8]. Therefore, the recent generalization [9] that 'ribose (deoxyribose) does not materially affect aggregation of nucleosides' is certainly not justified. Effects of phosphorylation were studied more extensively only for adenosine, with the main emphasis being placed on the geometry of the complexes formed at neutral pH [10–16] and the effects of protonation of adenine residues on association of nucleotides [17–19]. Strong electrostatic interactions between the positively charged adenine ring nitrogen atom and the ionized phosphate group of the adjacent nucleotide bring about a large increase in association.

In this paper we present results of fluorescence quenching studies on the self-association of 2-aminopurine riboside, its 5'-monophosphate and 5'-diphosphate in neutral aqueous solutions. Results of a similar study on self-association of the parent 2-aminopurine were published previously [20], along with a detailed description of the principles of the fluorescence method then developed. This method, because of its sensitivity and accuracy, allows interpretation of experimental data according to more reliable molecular models of association. The results of a parallel study of self-association of spin-labeled 2-aminopurine riboside and its 5'-monophosphate by the ESR method, recently developed and applied to associations of adenine nucleotides [19], are also presented and compared with those obtained from fluorescence quenching experiments.



Scheme 1.

2. Materials and methods

2-Aminopurine riboside (rn^{2}Pur) was obtained by reduction of 6-thioguanosine with Raney nickel [21]. Its 5'-monophosphate (P-rn^{2}Pur) and 5'-diphosphate (PP-rn^{2}Pur) were synthesized by phosphorylation of rn^{2}Pur according to refs. 22 and 23, respectively. The purity of the compounds was checked chromatographically in several solvent system. Their aqueous solutions appeared homogeneous with respect to the fluorescence lifetime, for the fluorescence quantum yield varied with concentration of the quencher, KBr, according to the Stern-Volmer relationship.

Derivatives of rn^{2}Pur and P-rn^{2}Pur spin labeled with the pyridoxal stable radical at the 2'(3')-OH group of the ribose residue were prepared as described previously [24] for adenine nucleotides.

Stock aqueous solutions of known concentrations of the studied compounds were prepared by weighing and then appropriately weight diluted. Experimental conditions (range of solute concentrations and temperatures investigated, ionic strength and pH of solutions) of fluorescence and ESR studies are given in table 1.

The apparatus and methods used in fluorescence studies have been described previously [20,25]. Fluorescence spectra were obtained by excitation with a xenon XBO 150 lamp at the absorption maximum at 304 nm, while fluorescence signals were recorded at an apparent emission maximum at 370 nm. Absorption spectra were measured with a Cary 118 spectrophotometer using an interference filter in order to cut off fluorescence from the samples.

ESR spectra were obtained with a model PE 1306 spectrometer, equipped with a storage accessory and a sample thermostat. Rotational correlation times τ_c of the spin label were derived from parameters of ESR spectra according to:

$$\tau_c = 6.5 \times 10^{-10} \cdot \Delta H_0 \left(\sqrt{\frac{h_0}{h_+}} + \sqrt{\frac{h_0}{h_-}} - 2 \right) \text{ s.}$$

where ΔH_0 is the width of the central component of the ESR spectrum in gauss, and h_+, h_0 and h_- the intensities of the ESR spectrum components corresponding to spin numbers of the nitrogen nucleus +1.0 and −1, respectively.

Calculations were performed with a Compucorp 425 G Scientists calculator and also on an Odra 1204 computer using a Mincon-20 programme [26,27] in a version without derivatives.

2.1. Fluorescence quenching method

Variation of the fluorescence quantum yield, ϕ, of a solute A with its concentration owing to formation of nonfluorescent excimers $^1A_2 \ldots {}^1A_n$ by (i) excitation of stacked complexes $A_2 \ldots A_n$ preformed in the ground state, and simultaneously (ii) in a diffusion-controlled reaction between an excited molecule ^1A and one(s) in the ground state, can be described [20] by:

$$\frac{\phi^0}{\phi} = 1 + \mathcal{K} C'(1-\gamma) \frac{[C_m + (C - C_m)\alpha]}{[C_m + (C - C_m)\alpha\gamma]} \tag{1}$$

in which ϕ^0 is the quantum yield of fluorescence of A at infinite dilution; $\mathcal{K} = \tau_0 k_e$, the quenching constant, where τ_0 is the natural fluorescence lifetime of the excited molecule ^1A and k_e an average rate constant of excimer formation; C, C' and C_m are stoichiometric, colligative and monomer concentrations of A, respectively; γ the probability of excimer dissociation, and α the hypochromicity coefficient.

C_m and C' are determined by the stoichiometric concentration C and association equilibrium constants, $K_n = [A_n]/C_m[A_{n-1}]$, defined according to an assumed model of molecular association. Parameters K_n, $\mathcal{K}$, γ, α, and ϕ^0 of eq. 1 can in principle be calculated by an iterative procedure, provided that the values of the $\phi = f(C)$ function have been found experimentally. Parameters K_n and $\mathcal{K}$ are strongly coupled [20], but their separation can be achieved by the use of KBr as fluorescence quencher, for Br^- shortens the τ_0 value of ^1A, thus decreasing $\mathcal{K}$ proportionally to the Br^- concentration. Comparison of the values of $\phi = f(C)$ and $\phi_q = f_q(C)$ functions, measured for solutions without and with the quencher (ϕ_q), leads to separation of the parameters, provided that all the others remain unaffected by the presence of KBr in the solution. It has been shown [20] that KBr up to 1.0 M does not alter the stacking equilibrium of 2-aminopurine, nor does Br^- associate with it.

The number of parameters, K_n, describing the stacking equilibrium which can be iteratively calculated depends on the accuracy of the experimental data and the range of concentrations investigated. It is necessary to assume a particular model of association in order to reduce realistically the number of K_n: (i) either an isodesmic model in which all K_n corresponding to the consecutive steps of indefinite polymerization are set equal to each other $K_2 = K_3 \ldots K_n$, or, at best, a model (ii) in which the dimerization constant K_2 differs from all the others set again equal. For the latter model, stoichiometric and colligative concentrations of A in solutions are described as follows:

$$C = \frac{\psi C_m}{(1 - K_p C_m)^2} - \psi C_m + C_m$$

$$C' = \frac{\psi C_m}{(1 - K_p C_m)} - \psi C_m + C_m$$

where K_p is the polymerization equilibrium constant and $\psi = K_2/K_p$ the cooperativity parameter. For the isodesmic model $\psi = 1$, and the above equations become simplified accordingly.

In order to derive thermodynamic functions related to the stacking association of a fluorophore, values of $\phi = f(C)$ and $\phi_q = f_q(C)$ functions are measured at various temperatures. $\phi(C, T)$ values determined for dilute solutions of A are then linearly extrapolated to $C = 0$ and the $\phi^0(T)$ values thus obtained are interpolated by fitting with a function of the form:

$$\phi^0 = \frac{[k_f/(k_f + k_{nr}^0)]}{[1 + \nu/(k_f + k_{nr}^0)\exp(-\Delta E/RT)]} \tag{2}$$

where k_f is the fluorescence rate constant, k^0_{nr} the sum of all temperature-independent rate constants of radiationless $A \leftarrow {}^1A$ transitions, ν the preexponential frequency factor and ΔE the activation energy. Constant parameters of eq. 2 are calculated iteratively.

Having determined independently the natural lifetime and fluorescence quantum yield ϕ^0 of the fluorophore at one temperature, values of $\tau_0 = (\phi^0)^{-1}$ at all. other temperatures are then calculated using eq. 2 and so are subsequently also the corresponding excimer formation rate constants $k_e = \mathcal{K}/\tau_0$. The latter are in turn interpolated according to the Noyes treatment [25] of fast processes, as described in detail previously [20]. In this treatment k_e can be expressed as the following function of temperature:

$$k_e = k_{diff}\left\{1 + \frac{k_{diff}}{\Phi p^2 (8\pi kT/\mu)^{1/2} \exp(-\Delta E/RT)}\right\}^{-1} \quad (3)$$

where Φ is a steric factor, μ the reduced mass of the reacting molecules, p the encounter distance and ΔE the activation energy. Whereafter, fluorescence quenching constants k_q are calculated, using the Stern-Volmer equation, and interpolated similarily to k_e. With the knowledge of k_q^{int} fluorescence quenching constants, $\mathcal{K}_q^{int}$ can be calculated from the relationship $\phi^0/\phi^0_q = \mathcal{K}/\mathcal{K}_q$. Interpolated values of ϕ^{int} and $\mathcal{K}_q^{int}$ are then used to calculate from eq. 1 the final values of $K_n(T)$ and/or $K_2(T)$ and $K_p(T) = K_2(T)/\psi(T)$ equilibrium association constants. Fitting of the latter with either a linear van't Hoff equation or, if necessary, the corresponding ΔG with a higher order function of temperature leads to determination of the enthalpy and entropy of the stacking association.

2.2. *ESR spin-label method*

Formation of associates between spin-labeled A^+ and unlabeled A molecules brings about a decrease in the rotational diffusion frequency ν_{exp} of the spin label, derived from parameters of the ESR spectrum. Provided that the stoichiometric concentration of spin-labeled molecules C_{A^+} in solution is kept sufficiently low as compared with that of unlabeled ones ($C_{A^+} \ll C_A$) and that only complexes of the type $A_nA_1^+$ can be considered, then $\nu_{exp} = \sum_{n=1}^{\infty} \nu_n f_n$, where ν_n and f_n are the rotational diffusion frequency and the relative fraction of an associated species of the type $A_nA_1^+$, respectively. By assuming the isodesmic model of association characterized by one equilibrium association constant K_{isod} and one average rotational diffusion frequency ν_{ass} for all $A_nA_1^+$ complexes, it was shown that the dependence of ν_{exp} on the stoichiometric concentration of compound A can be described [19] by the following analytical equation:

$$\left\{\frac{(\nu_1 - \nu_{exp})}{C_A}\right\}^{1/2} = \left\{\frac{K_{isod}}{(\nu_1 - \nu_{ass})}\right\}^{1/2} [(\nu_1 - \nu_{ass}) - (\nu_1 - \nu_{exp})] \quad (4)$$

where ν_1 is the rotational diffusion frequency of a spin-labeled monomer. From the plot of $\{(\nu_1 - \nu_{exp})/C_A\}^{1/2}$ versus $(\nu_1 - \nu_{exp})$ the two unknown parameters K_{isod} and ν_{ass} can be determined.

3. Results

3.1. *Fluorescence studies*

Fluorescence quantum yields, ϕ, of m^2Pur, *P*-m^2Pur and *PP*-m^2Pur were measured for two series of neutral aqueous solutions, one of which contained 0.2 M KBr as the fluorescence quencher, as a function of solute concentration and temperature under the experimental conditions specified in table 1. The ionic strength of solutions of both nucleotides was kept high and constant at $I = 0.8$ in order to shield effectively negative charges on the phosphate groups and thereby to minimize electrostatic repulsion between the solute molecules, expected to affect stacking of their base residues, as observed recently for adenine nucleotides [19].

It was found that quenching of fluorescence by Br^- in solutions of all three compounds investigated obeyed the Stern-Volmer relationship up to 0.8 M KBr, both at low and high solute concentrations. This observation indicated that the fluorescence of molecules in stacked complexes is fully quenched so that the probability of excimer dissociation, γ, in eq. 1 can be set equal to zero. The

Table 1

Experimental conditions of fluorescence quantum yield $\phi(C, T)$ and ESR spectra determination

Compound	Method	Solution			Temperature range [b] (K)
		Range of solute concentrations [b] (M)	Electrolyte concentration (M)	pH [a]	
rn^{2}Pur	fluorescence	5×10^{-4}–9×10^{-2} (15)	none	7.0–7.5	278–358 (10)
		5×10^{-4}–9×10^{-2} (15)	KBr, 0.2	7.0–7.5	278–358 (10)
P-rn^{2}Pur	fluorescence	8×10^{-5}–9×10^{-3} (21)	KCl, 0.8	7.0–7.5	276–358 (19)
		8×10^{-4}–9×10^{-3} (16)	KCl, 0.6 KBr, 0.2	7.0–7.5	276–358 (19)
PP-rn^{2}Pur	fluorescence	1×10^{-4}–3×10^{-2} (19)	KCl, 0.8	7.0–7.5	276–358 (19)
		1×10^{-4}–3×10^{-2} (19)	KCl, 0.6 KBr, 0.2	7.0–7.5	276–358 (19)
rn^{2}Pur spin labeled	ESR	1×10^{-2}–1.2×10^{-1} (11)	none	7.5	277–325 (10)
P-rn^{2}Pur spin labeled	ESR	1×10^{-2}–1.2×10^{-1} (11)	KCl, 0.6 KBr, 0.2	7.5	277 and 305
			NaCl, 0.1	7.5	276–334 (12)

[a] Measurements of $\phi(C, T)$ at $\lambda = 322$ nm (isosbestic point) indicated that it remains constant in the pH range 6.4–9.2 for *P*-rn^{2}Pur and *PP*-rn^{2}Pur solutions; without buffer.

[b] The number of solutions or temperatures investigated are given in parentheses.

hypochromicity coefficients were evaluated using as an initial value $\alpha = 0.97$ determined for 2-aminopurine (n^{2}Pur) stacking association [20]. In trial calculations the following values were found to introduce the least systematic errors not exceeding 2.5% into K_n values: $\alpha = 0.95$ for rn^{2}Pur and $\alpha = 0.90$ for both nucleotides. They were used in further calculations as established parameters. The parameters ϕ^0, K_n and $\mathcal{K}$ were then calculated iteratively by fitting the function eq. 1 to the experimental $\phi(C, T)$ data, as described in section 2.1. Empirical parameters of the functions (eqs. 2 and 3) used for interpolation of $\phi^0(T)$, $k_e(T)$ and $k_q(T)$ data are shown in table 2. Values of fluorescence quantum yields ϕ^0 were determined relative to the quantum yield of the parent 2-aminopurine, $\phi^0 = 0.844$, in aqueous solution at 20.1°C [20]. In view of the close similarity of the absorption and emission spectra of 2-aminopurine and all its three derivatives studied, the same value of the fluorescence rate constant $k_f = 6.95 \times 10^7$ s^{-1}, as found for n^{2}Pur [20], was assumed in calculations of their natural fluorescence lifetimes $\tau_0(T)$.

Both models of association defined in section 2.1 were tried in computations. The two-parameter model (ii) gave distinctly better results for rn^{2}Pur. Fitting of either of the two models to the experimental data for *PP*-rn^{2}Pur was equally satisfactory, while in the case of *P*-rn^{2}Pur only the isodesmic one (i) was applied, because of the relatively narrow range of the solute concentrations investigated.

The calculated K_2 and $K_p = K_2/\psi$ values for rn^{2}Pur and the equilibrium constants obtained according to the isodesmic model, K_i, for *P*-rn^{2}Pur and *PP*-rn^{2}Pur association are shown in tables 3

Table 2

Parameters of the functions (eqs. 2 and 3) used for interpolation of $\phi^0(T)$, $k_e(T)$ and $k_\eta(T)$ data

Compound	$\phi^0 = a/[1 + a' \exp(-\Delta E/RT]$			$k = b(T\eta^{-1})/[1 + b'T^{1/2}\eta^{-1}) \exp(-\Delta E/RT)]$					
				$k = k_e$			$k = k_\eta$		
	a	a'	ΔE (kcal mol^{-1})	b	b'	ΔE (kcal mol^{-1})	b	b'	ΔE (kcal mol^{-1})
m^2Pur	1.103	814.4	−5.014	2.465×10^{-6}	3×10^5	−3.99	1.032×10^5	8.444×10^{-4}	0
P-m^2Pur	1.119	2.634×10^3	−4.99	1.107×10^{-6}	3×10^5	−5.0	9.716×10^4	2.257×10^{-4}	−0.662
PP-m^2Pur	1.107	2.998×10^4	−6.64	1.054×10^{-5}	3×10^5	−3.4	8.831×10^4	3.463×10^{-4}	−0.408

Table 3

Experimental and interpolated (int) dimerization (K_2) and polymerization (K_p) equilibrium constants of rn^{2}Pur self-association K_2^{int} calculated from the equation: $\Delta G_2 = 2007.9 - 28.33T + 0.058867T^2$; K_p^{int} calculated from the equation: $\Delta G_p = 35300 - 259.51T + 0.457T^2$.

Temperature (K)	K_2 (l mol^{-1})	K_2^{int} (l mol^{-1})	$\Delta K_2/K_2^{int}$ (%)	K_p (l mol^{-1})	K_p^{int} (l mol^{-1})	$\Delta K_p/K_p^{int}$ (%)
278.78	11.098	10.741	2.7	15.473	15.033	2.8
282.43	10.334	10.102	2.2	14.094	14.801	−5.0
295.19	7.683	8.080	−5.2	11.904	11.960	−0.5
304.26	6.536	6.840	−4.6	9.122	8.944	1.9
313.32	5.738	5.757	−0.3	6.361	6.032	5.2
322.35	5.020	4.822	3.9	3.696	3.715	−0.5
334.96	4.019	3.829	4.5	1.602	1.626	−1.5
343.93	2.991	3.097	−3.6	0.827	0.825	0.3
352.88	2.489	2.560	−2.9	0.376	0.391	−3.9
358.20	2.327	2.281	2.0	0.246	0.243	1.2

Table 4

Experimental and interpolated (int) isodesmic equilibrium constants (K_i) of P-rn^{2}Pur and PP-rn^{2}Pur self-association, and related thermodynamic functions

Temperature (K)	P-rn^{2}Pur			PP-rn^{2}Pur		
	K_i (l mol^{-1})	K_i^{int} (l mol^{-1})	$\Delta K_i/K_i^{int}$ (%)	K_i (l mol^{-1})	K_i^{int} (l mol^{-1})	$\Delta K_i/K_i^{int}$ (%)
276.96	9.41	8.20	14.80	6.17	5.47	12.69
282.44	7.60	7.87	−3.48	5.26	5.11	2.94
286.09	7.40	7.66	−3.49	4.90	4.89	0.16
291.56	6.91	7.38	−6.29	4.40	4.58	−4.01
295.19	6.96	7.20	−3.36	4.26	4.40	−3.23
300.65	6.48	6.94	−6.68	3.94	4.14	−4.85
304.27	6.83	6.78	0.67	3.79	3.98	−4.92
309.71	6.46	6.56	−1.43	3.57	3.76	−5.05
313.33	6.66	6.41	3.80	3.49	3.63	−3.89
318.75	6.22	6.22	0.00	3.35	3.44	−2.78
322.36	6.39	6.09	4.86	3.37	3.32	1.41
327.77	6.03	5.91	2.10	3.16	3.16	0.00
331.37	6.04	5.80	4.18	3.15	3.06	2.83
334.97	5.26	5.69	−7.49	2.95	2.96	−0.68
340.35	5.77	5.53	4.19	3.01	2.83	6.36
343.94	5.69	5.43	4.60	2.89	2.75	5.09
349.91	5.51	5.30	4.05	2.62	2.63	0.06
352.89	4.84	5.21	−7.03	2.59	2.55	1.40
358.25	5.01	5.08	−1.26	2.41	2.45	−1.76
ΔH_i (van't Hoff) $= -1.0 \pm 0.1$ kcal mol^{-1} $\Delta S_i = 0.0 \pm 0.3$ e.u.				ΔH_i (van't Hoff) $= -2.0 \pm 0.1$ kcal mol^{-1} $\Delta S_i = -3.7 \pm 0.3$ e.u.		

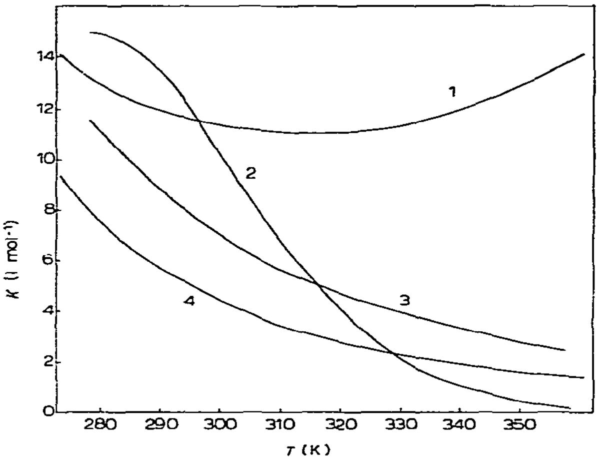


Fig. 1. Temperature dependence of equilibrium constants for dimerization (K_2) and polymerization (K_p) steps of n^{2}Pur [20] and rn^{2}Pur self-association in water. Curves: (1) $K_p(T)$, n^{2}Pur; (2) $K_p(T)$, rn^{2}Pur, (3) $K_2(T)$, rn^{2}Pur; (4) $K_2(T)$, n^{2}Pur.

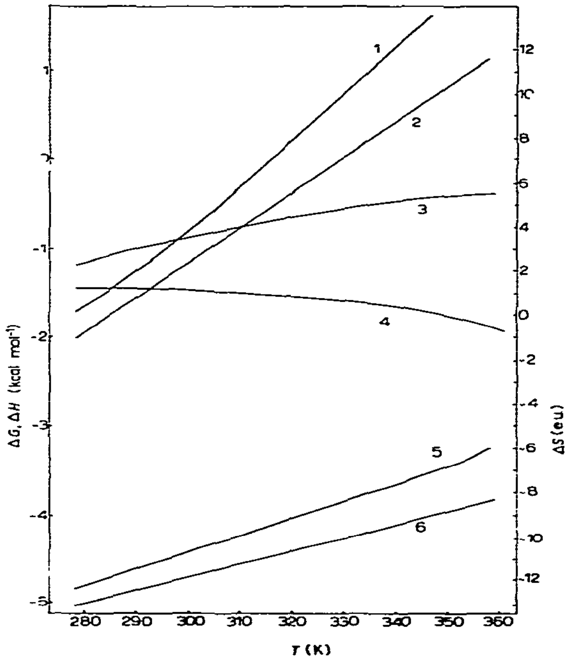


Fig. 2. Variation with temperature of thermodynamic function of n^{2}Pur self-association in water, calculated from the equations [20]: $\Delta G_2 = -7213 + 30.3T - 0.0308T^2$ (dimerization step): $\Delta G_p = -7893.5 + 45.396T - 0.07971T^2$ (polymerization step). Curves: (1) $\Delta H_p(T)$, (2) $\Delta S_p(T)$, (3) $\Delta G_2(T)$, (4) $\Delta G_p(T)$, (5) $\Delta H_2(T)$, (6) $\Delta S_2(T)$.

and 4 along with the respective interpolated values. The latter were obtained by numerical interpolation assuming that in the case of rn^{2}Pur the free enthalpy of association can be correctly described by a second-order function of temperature, while for P-rn^{2}Pur and PP-rn^{2}Pur K_i values vary with temperature according to the van't Hoff equation.

We shall first consider the effects of N(9) substitution with ribose on the stacking association of n^{2}Pur. As seen from fig. 1, in the case of n^{2}Pur autoassociation the process is cooperative at all temperatures ($K_p > K_2$), while autoassociation of rn^{2}Pur is cooperative only at $T < 318$ K and at higher temperatures becomes anticooperative ($K_p < K_2$). The mode of rn^{2}Pur autoassociation thus depends on temperature. As a consequence, $K_p = f_p(T)$ functions describing the temperature dependence of the polymerization step of self-association of n^{2}Pur [20] and its riboside, nr^{2}Pur, differ appreciably, indicating that the underlying mechanisms of association should also be different. Comparison of the K_2 values shows that in the case of the riboside association the dimerization step is at all temperatures more favorable. The similarity of the $K_2 = f_2(T)$ functions might at first sight suggest that the molecular organization of n^{2}Pur and rn^{2}Pur dimeric species is similar. This is, however, clearly not the case, since the plots of ΔH_2 and ΔS_2 thermodynamic functions versus temperature (figs. 2 and 3) demonstrate that $\partial \Delta H_2/\partial T$ and $\partial \Delta S_2/\partial T$ derivatives of the respective functions have opposite signs. This observation holds also for pairs of $\partial \Delta H_p/\partial T$ and $\partial \Delta S_p/\partial T$ derivatives. For a given compound the signs of $\partial \Delta H/\partial T$ and $\partial \Delta S/\partial T$ functions are the same for both dimerization and polymerization steps. It may be thus safely concluded that the molecular mechanisms of formation of dimeric and polymeric species involved in self-association of n^{2}Pur and rn^{2}Pur must differ appreciably.

It is worth examining in this connection the thermodynamic data for self-association of other

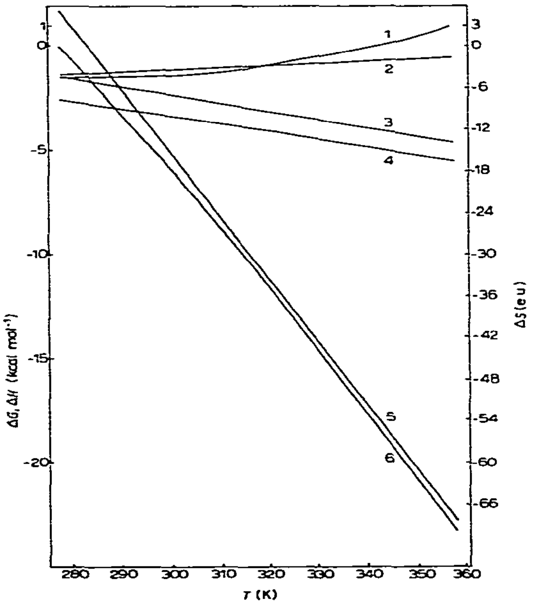


Fig. 3. Variation with temperature of thermodynamic functions of rn^{2}Pur self-association in water, calculated from the equations: $\Delta G_2 = 2007.9 - 28.33T + 0.0589T^2$ (dimerization step): $\Delta G_p = 35300 - 259.5T + 0.457T^2$ (polymerization step). Curves: (1) $\Delta G_p(T)$, (2) $\Delta G_2(T)$, (3) $\Delta S_2(T)$, (4) $\Delta H_2(T)$, (5) $\Delta S_p(T)$, (6) $\Delta H_p(T)$.

nucleic acid bases and their corresponding nucleosides. Inspection of the available association equilibrium constants and related thermodynamic parameters (table 5), derived from vapor pressure osmometric studies according to the isodesmic model of association and by fitting the van't Hoff equation to the equilibrium data, leads to the conclusion that replacement of a hydrogen atom or a methyl group bound to a ring nitrogen by ribose (deoxyribose), both in diketopyrimidines and purines, brings about similar changes in the enthalpy and entropy of association. Namely, for nucleosides they become distinctly less negative as compared with those characteristic of association of free bases. In most cases this results also in a somewhat lower tendency of nucleosides to association at 298 K. Except for the latter point, substitution of n^{2}Pur with ribose results in similar changes in thermodynamic parameters for the dimerization step at the same temperature. These observations are clearly in contrast to the conclusion expressed in a recent review article [9] that ribose or deoxyribose do not materially affect aggregation of nucleosides.

Before we try to rationalize the thermodynamic data for rn^{2}Pur self-association in terms of the possible molecular structure of the complexes

Table 5

Effects of ribose (deoxyribose) substitution on equilibrium constant (K_i, isodesmic model) at 298 K and thermodynamic functions of autoassociation of nucleic acid bases in aqueous solution

Compound	K_i^{298} (molal^{-1})	ΔH^0 (van't Hoff) (kcal mol^{-1})	ΔS^0 (e.u.)	Reference
m^{1}Ura	0.83	−5.2	−17.8	7
rUrd	0.64	−2.1	−7.8	7
m^{3}Ura	1.13	−4.9	−16.2	7
rm^{3}Urd	0.65	−2.7	−10.1	8
m^{1}Thy	0.93	−4.1	−13.9	7
dThd	1.03	−2.6	−8.7	7
Pur	2.1	−4.2	−13	38
rPuo	1.91	−2.5	−7	38
n^{2}Pur	4.7 [a]	−4.5 [b]	−11.9 [b]	this work
rn^{2}Pur	7.65 [a]	−3.2 [b]	−6.8 [b]	

[a] Corresponds to dimerization equilibrium constant K_2.

[b] At 298.15 K, temperature-dependent thermodynamic functions.

formed, it is first necessary to recall that by virtue of the fluorescence quenching method employed, only formation of associates involving stacking of the bases has been measured. This is not necessarily the case when association is measured by techniques based on other molecular properties, particularly on colligative properties like osmotic pressure.

In the case of free n^2Pur base self-association, a small negative enthalpy and a positive entropy of polymerization at 293 K were interpreted previously [20] as being due to an important hydrophobic contribution to stabilization of some higher-order stacked complexes. Plots of ΔH_p and ΔS_p versus temperature (cf. fig. 2) demonstrate that this contribution increases with rise of temperature, in agreement with the Nemethy-Scheraga model of hydrophobic interactions [29]. A hidden hydrophobic contribution to the thermodynamic parameters of the dimerization step can be also inferred from the positive sign of $\partial\Delta H_2/\partial T = \bar{C}_{p2}$ and $\partial\Delta S_2/\partial T$ derivatives. The increase with temperature of the partial heat capacity in aqueous solutions, of nucleic acid bases n^2Pur and adenine derivatives inclusive, has been measured calorimetrically [4,30].

The presence of the ribose residue in rn^2Pur is expected to affect the stacking geometry of the bases in associated species and may in addition bring about direct and/or water-bridge-mediated hydrogen-bond interactions between the furanose hydroxyl moieties of adjacent molecules, accompanied by changes in their hydration shells. Because of the difference between the bases hydration properties and those of sugar residues which are hydrophilic in nature, the thermodynamics of reorganization of their hydration shells upon association can also be expected to differ from those observed for stacking of unsubstituted bases. Unfortunately, there is as yet insufficient knowledge about the thermochemical properties of carbohydrate monomers in aqueous solutions to allow any sensible predictions concerning changes in thermodynamic functions due to their association [31].

For the sake of simplification of the ensuing discussion let us assume that the experimental enthalpies and entropies of nucleoside association can be represented as additive contributions arising from: (i) stacking association of the bases, ΔH_{bb} and ΔS_{bb}; (ii) hydrogen-bond interactions between sugar furanose rings, ΔH_{ass} and ΔS_{ss}; and (iii) changes in the hydration shells of these two nucleoside constituents, ΔH_{hb}, ΔS_{hb}, and ΔH_{hs}, ΔS_{hs}, respectively.

The number of mutual geometric orientations accessible in the process of vertical base-base association of nucleosides is necessarily somewhat limited by the presence of bulky sugar moieties. It is thus possible that some configurations of the bases within stacked complexes, corresponding to the lowest negative enthalpies of their formation ($\Delta H_{bb} < 0$), may become sterically excluded, while those characterized by a higher contribution from hydrophobic interactions ($\Delta H_{hb} \leqslant 0$, $\Delta S_{hb} > 0$) are favored at the same time. This would result in a smaller experimental enthalpy of the nucleoside association than that of the corresponding free base. In this way the differences in thermodynamic data for the dimerization step of n^2Pur and rn^2Pur as well as between those for association of other free bases and their nucleosides, shown in table 5, can at first sight be rationalized. It should be noted that because of the relatively low concentrations of solutes used and the small equilibrium association constants of pyrimidine bases, the data in table 5 are to a large extent related to formation of dimeric species.

Ribose itself does not exhibit any appreciable tendency to self-association for its osmotic coefficient in water is equal to unity up to 4 M concentration whereas xylose shows measurable solute interactions [32], as do also some other polyhydroxy compounds. It is thus highly probable that the detailed molecular stereochemistry of polyhydroxy compounds determines their hydration and association properties [33]. Therefore, ribose in the form of a closed β-D-furanose ring, which it attains in nucleosides, may have quite different solution properties. Thus, once a nucleoside stacked dimer is formed, hydrogen-bond interactions between adjacent furanose moieties may add cooperatively, thereby inducing a rearrangement of the complex towards attainment of a more thermodynamically favorable and highly packed configuration in which both the base and ribose residues overlap extensively. One can envisage several such configurations as being sterically possible, particularly for purine nucleosides in which internal rotation around the glycosidic C–H bond is relatively free. Such rearrangement of hydrogen-bonded structures, accompanied by concomitant relaxation of the solvent shells around specifically hydrated sugar residues, cannot be athermal as in the case of amide association [34,35], but is rather exothermic ($\Delta H_{ss} + \Delta H_{hs} < 0$). It results namely in formation of distinctly weaker hydrogen bonds between sugar hydroxyl groups ($pK \approx 12-13$) than those involved in their hydration and interaction between water molecules themselves, after their removal from sugar surfaces in the process of nucleoside association. As seen from fig. 3, the experimental total enthalpy of association of rn^2Pur is dominated by the ΔH_p term and compared with that of n^2Pur association it is indeed more negative at all temperatures, while the sign of $\partial\Delta H_2/\partial T$ and $\partial\Delta H_p/\partial T$ functions supports the supposition that the most important enthalpic contribution to ΔG_{ass} is connected with changes in hydrophilic hydration of sugar residues. The negative sign of $\partial\Delta S_2/\partial T$ and $\partial\Delta S_p/\partial_T$ functions also indicates that negative entropy of the rn^2Pur association, particularly beyond the dimer stage, is dominated by the negative ΔS_{hs} term. At the dimer stage, ΔG_2 apparently contains still important enthalpic and entropic contributions determined by stacking of n^2Pur base residues at such a selected geometry(ies) that $|\Delta H_{bb} + \Delta H_{hb}|_{\text{nucleoside}} < |\Delta H_{bb} + \Delta H_{hb}|_{\text{free base}}$ and $|\Delta S_{bb} + \Delta S_{hs}|_{\text{nucleoside}} < |\Delta S_{bb} + \Delta S_{hb}|_{\text{free base}}$.

Substitution of rn^2Pur at the C-5'-OH of ribose with a negatively charged ester group, viz., monophosphate or diphosphate, greatly affects the thermodynamics of association of respective ribonucleotides, involving stacking of their n^2Pur base residues. As seen from tables 3 and 4, the tendency to association at 298 K, measured by free enthalpies of association ΔG, decreases in the order: rn^2Pur > *P*-rn^2Pur > *PP*-rn^2Pur. At the same time, the absolute values of both enthalpic and entropic contributions to ΔG become significantly reduced as compared with those of the parent rn^2Pur. The values of the latter two thermodynamic functions at 298 K for *P*-rn^2Pur and *PP*-rn^2Pur seem to resemble rather those of n^2Pur free base polymerization. This can be taken as indirect evidence that association of both nucleotides is to a large extent controlled by stacking interactions of their n^2Pur residues. Such interpretation of the thermodynamic data remains in agreement with general considerations on the possible molecular organization of stacked complexes formed between nucleotides and finds additional support in the results of earlier studies on association of adenine nucleotides [19].

Under our experimental conditions of high ionic strength of nucleotide solutions, positive contribution to the free enthalpy of association from electrostatic interactions is minimized as a result of effective shielding of the negatively charged phosphate groups by counterions. It can be expected to remain still somewhat larger for *PP*-rn^2Pur than for *P*-rn^2Pur complexes. From this point of view, the most favorable organization of complexes would be such a one in which the bases attain head-to-tail stacked geometry while the esterified ribose residues become *trans* oriented so that they point in opposite directions. This type of packing was suggested for complexes of adenine nucleotides at neutral pH on the basis of ^{1}H-NMR studies of AMP association [10–13] and ultraviolet absorption and CD studies in the case of ATP [14]. Evans and Sarma [13] performed detailed comparative studies of ^{1}H-NMR chemical shifts, using fast Fourier-transform spectroscopy, on AMP association and the conformation of ApA and poly(A) in water. They concluded therefrom that though there are several unique time-averaged orientations of AMP in stacked complexes, only one, characteristic of the conformation and packing of adenine ribooligonucleotide single chains, seems more favorable than the others: namely, the one in which bases stack face to back while the ribose moieties of the adjacent molecule are near one another and the phosphate groups remain well separated. It is, of course, impossible to distinguish on the basis of thermodynamic data alone, which, if any, of the stereochemically possible complexes is more favorable in the case of n^2Pur nucleotides.

The observed temperature independence of ΔH_i and ΔS_i functions for association of the two n^2Pur nucleotides seems to be more apparent than real. These thermodynamic functions are certainly composed of several contributions which can be somewhat arbitrarily related to various types of intramolecular interactions within complexes. So that $\partial \Delta H_i/\partial T = 0$ and $\partial \Delta S_i/\partial T = 0$ result most probably from compensation of opposite temperature dependences of constituent terms. In addition to those enumerated in the case of rn²Pur, terms describing changes in hydration of ionic groups of nucleotides during association, ΔH_{hi} and ΔS_{hi}, should also be considered because anions of orthophosphoric acid are known to be strong stabilizers of water structure [36,37] and thereby may stabilize stacks of bases. The larger absolute values of ΔH_i and ΔS_i functions for *PP*-rn²Pur than those for *P*-rn²Pur can thus in part reflect the stronger influence of the triply charged diphosphate group on stacking of n²Pur bases and hydration of the whole complex as well. In this connection, however, it is necessary to note that in the light of ESR investigations of association of spin-labeled AMP, ADP and ATP adenine nucleotides in water at neutral pH and high ionic strength [19], the isode-

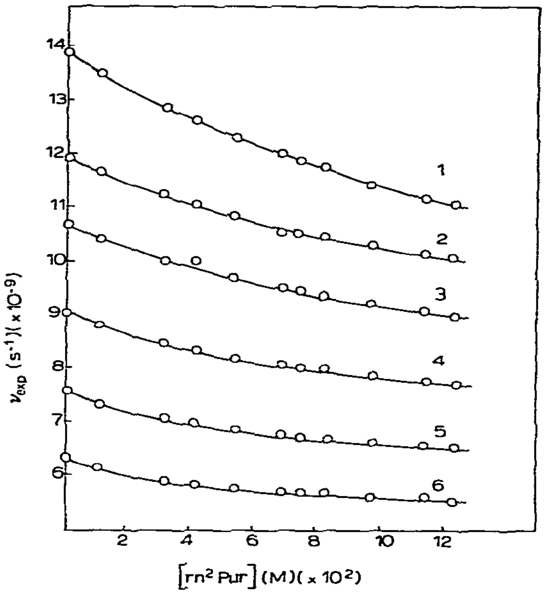


Fig. 4. Concentration dependence of diffusion rotational frequency ν_{exp} of spin-labeled rn²Pur at various temperatures. Curves: (1) 331 K, (2) 318 K, (3) 309 K, (4) 297 K, (5) 285 K, (6) 277 K.

Table 6

Experimental and interpolated (int) isodesmic equilibrium constants (K_i) of spin-labeled rn²Pur and *P*-rn²Pur self-association, and related thermodynamic functions

Spin-labeled rn²Pur				Spin-labeled *P*-rn²Pur			
Temperature (K)	K_i (l mol^{-1})	K_i^{int} (l mol^{-1})	$\Delta K_i/K_i^{int}$ (%)	Temperature (K)	K_i (l mol^{-1})	K_i^{int} (l mol^{-1})	$\Delta K_i/K_i^{int}$ (%)
277	7.12	7.10	0.28	276	8.74 (9.8)[a]	8.42	3.71
282	6.18	6.09	1.38	280	8.18	7.99	2.30
285	5.68	5.57	1.87	285	7.15	7.50	−4.98
290	4.79	4.83	−0.81	289	7.11	7.15	−0.59
297	3.87	3.97	−2.58	295	6.69	6.66	0.50
302	3.56	3.48	2.41	299	5.90	6.36	−7.77
309	2.81	2.91	−3.59	305	6.07 (7.1)[a]	5.96	2.16
313	2.50	2.64	−5.23	310	5.85	5.65	3.47
318	2.38	2.34	1.84	316	5.29	5.31	−0.45
325	2.09	1.99	4.86	321	4.99	5.05	−1.32
				327	4.95	4.77	3.55
				334	4.41	4.47	−1.34
$\Delta H_i = -4.73$ kcal mol^{-1} (van't Hoff)				$\Delta H_i = -1.98$ kcal mol^{-1} (van't Hoff)			
$\Delta S_i = -13.2$ e.u.				$\Delta S_i = -2.89$ e.u.			

[a] In 0.6 M KCl+0.2 M KBr.

Table 7

List of equilibrium constants and thermodynamic functions of self-association of n^2Pur, rn^2Pur, P-rn^2Pur and PP-rn^2Pur obtained from fluorescence quenching and ESR spin-label experiments

Compound	Fluorescence			ESR		
	K ($l\ mol^{-1}$) ($T=298.15$ K)	ΔH^0 (kcal mol^{-1})	ΔS^0 (e.u.)	K_i ($l\ mol^{-1}$) ($T=298.15$ K)	ΔH^0 (kcal mol^{-1})	ΔS^0 (e.u.)
n^2Pur	4.7 (K_2)	-4.48	-11.90			
	11.45 (K_p)	-0.81	$+2.14$			
rn^2Pur	7.65 (K_2)	-3.20	-6.80	3.8	-4.73	-13.2
	11.5 (K_p)	-5.32	-13.01			
P-rn^2Pur	7.1 (K_i)	-1.26	-0.35	6.6	-1.98	-2.89
PP-rn^2Pur	4.2 (K_i)	-1.95	-3.7			

smic equilibrium constant K_i apparently does not depend on the number of phosphate groups.

3.2. ESR studies; comparison of ESR and fluorescence data

Rotational diffusion frequencies ν_{exp} of spin-labeled rn^2Pur and P-rn^2Pur as a function of solute concentration and temperature (fig. 4) were obtained from ESR spectra measured under the experimental conditions specified in table 1. The isodesmic association equilibrium constants, derived according to eq. 4 (table 6), proved to vary with temperature according to the van't Hoff relationship.

The thermodynamic functions calculated therefrom (table 6) agree generally with the respective data obtained from fluorescence quenching studies (tables 3, 4 and 7). However, some differences are observed between the absolute values of particular functions obtained for a given compound by the two methods. They can be attributed to the presence of the nitroxide stable radical either at the ribose 2'- or 3'-OH group and can be explained in connection with the previously proposed model of intramolecular organization of association complexes.

Under the experimental conditions employed, viz., $C_{A^*} \ll C_A$, only formation of rn^2Pur complexes of the type $A_1A_1^+ \ldots A_nA_1^+$ should be taken into consideration. In these complexes direct hydrogen-bond interactions between ribose residues of the type postulated before seem rather improbable because of the bulkiness and different chemical nature of the nitroxide radical. This should be particularly significant for $A_1A_1^+$ dimeric species which, at the relatively low solute concentrations used in our studies, represent the largest fraction of associates. The lack of these interactions in complexes bearing spin-labeled rn^2Pur molecules is probably the main cause of the smaller free enthalpy of association obtained by ESR than by the fluorescence method. By the same token, one would not expect any appreciable difference to occur between free enthalpies of P-rn^2Pur association obtained by the two methods, provided that nucleotide molecules in stacked complexes attain the geometry discussed in the preceding section. Inspection of the thermodynamic data in table 7 shows that this indeed seems to be the case.

In the light of the above discussion, application of the ESR method is advisable in investigations in which possible interference due to the presence of a spin label can be reasonably excluded. Association of P-rn^2Pur as well as of the adenine nucleotides referred to above is a good example of its usefulness.

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References

1 P.O.P. Ts'o, Basic principles of nucleic acids chemistry, vol. 1 (Academic Press, New York, 1974), p. 537.
2 R. Lawaczek and K.G. Wagner, Biopolymers 13 (1974) 2003.
3 K.G. Wagner, in: Nuclear magnetic resonance in molecular biology, ed. B. Pullman (D. Reidel, Dordrecht, 1978) p. 103.
4 J. Szemińska, W. Zielenkiewicz and K.L. Wierzchowski, Biophys. Chem. 10 (1979) 409.
5 A. Zielenkiewicz, E. Plesiewicz and K.L. Wierzchowski, Biophys. Chem. 10 (1979) 415.
6 A.B. Teplitsky, I.K. Yanson, O.T. Glukhova, A. Zielenkiewicz, W. Zielenkiewicz and K.L. Wierzchowski, Biophys. Chem. 11 (1980) 17, 475.
7 E. Plesiewicz, E. Stępień, K. Bolewska and K.L. Wierzchowski, Biophys. Chem. 4 (1976) 131.
8 E. Plesiewicz, E. Stępień, K. Bolewska and K.L. Wierzchowski, Nucleic Acid Res. 3 (1976) 1295.
9 F. Franks, in: Biochemical thermodynamics, ed. M.N. Jones (Elsevier, Amsterdam, 1979) p. 70.
10 M.P. Schweitzer, A.D. Brown, P.O.P. Ts'o and D.P. Hollis, J. Am. Chem. Soc. 90 (1968) 1042.
11 M. Gueron, C. Chachaty and T.-D. Son, Ann. N.Y. Acad. Sci. 222 (1973) 307.
12 T.-D. Son and C. Chachaty, Biochim. Biophys. Acta 335 (1973) 1.
13 F.E. Evans and R.H. Sarma, Biopolymers 13 (1974) 2117.
14 M.P. Heyn and R. Bretz, Biophys. Chem. 3 (1975) 35.
15 W. Egan, J. Am. Chem. Soc. 98 (1976) 4091.
16 P.R. Mitchell and H. sigel, Eur. J. Biochem. 88 (1978) 149.
17 W.E. Fergusson, C.M. Smith, E.T. Adams, Jr and G.I. Barlow, Biophys. Chem. 1 (1974) 325.
18 T.J. Gilligan, III and G. Schwartz, Biophys. Chem. 4 (1976) 55.
19 A.I. Petrov and B.I. Sukhorukov, Mol. Biol. 14 (1980) 439.
20 A. Bierzyński, H. Kozłowska and K.L. Wierzchowski, Biophys. Chem. 6 (1977) 213.
21 J.J. Fox, I. Wempen, A. Hampton and I.L. Doerr, J. Am. Chem. Soc. 80 (1980) 1669.
22 J. Giziewicz and D. Shugar, in: Nucleic acid chemistry, eds. L.B. Townsend and R.S. Tipson (J. Wiley, New York, 1978) p. 955.
23 C. Janion and D. Shugar, Acta Biochem. Pol. 20 (1973) 271.
24 A.I. Petrov and B.I. Sukhorukov, Biofizika 20 (1975) 965.
25 A. Bierzyński and J. Jasny, J. Photochem. 3 (1974/75) 431.
26 R. Fletcher and M.J.D. Powell, Comput. J. 6 (1963) 163.
27 M.J.D. Powell, Comput. J. 7 (1964) 155.
28 R.M. Noyes, in: Progress in reaction kinetics, vol. 1, ed. G. Porter (Pergamon Press, London, 1961) p. 129.
29 G. Nemethy and H.A. Scheraga, J. Phys. Chem. 66 (1962) 1773.
30 J. Szemińska, A. Zielenkiewicz and K.L. Wierzchowski, in preparation.
31 D.S. Reid, in: Biochemical thermodynamics, ed. M.M. Jones (Elsevier, Amsterdam, 1979) p. 173.
32 F. Franks, in: Biochemical thermodynamics, ed. M.M. Jones (Elsevier, Amsterdam, 1979) p. 65.
33 A. Suggett, in: Water, vol. 4, ed. F. Franks (Plenum Press, New York, 1975) p. 519.
34 D.M. Klotz and S.B. Farnham, Biochemistry 7 (1968) 3879.
35 G.S. Kresheck and D.M. Klotz, Biochemistry 8 (1969) 8.
36 P.H. von Hippel and T. Schleich, in: Structure and stability of biological macromolecules, eds. S.N. Timasheff and G.D. Fasman (Marcel Dekker, New York, 1969) p. 417.
37 D.R. Robinson and M.E. Grant, J. Biol. Chem. 241 (1966) 4030.
38 S.J. Gill, M. Downing and G.F. Sheats, Biochemistry 6 (1967) 272.