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OSMOMETRIC STUDIES ON SELF-ASSOCIATION OF PYRIMIDINES IN AQUEOUS SOLUTIONS: EVIDENCE FOR INVOLVEMENT OF HYDROPHOBIC INTERACTIONS

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Vapour pressure osmometric studies were performed on stacking self-association of 25 uracil derivatives variously C- and N-substituted with polar and alkyl groups in aqueous solution at various temperatures. The respective equilibrium association constants K_{st} were computed on the assumption of the isodesmic model of self-association ($K_2 = K_3 = ... = K_n = K_{st}$). Enthalpies of association for most of the compounds studied were obtained from the temperature-dependence of K_{st}, according to the van 't Hoff equation. Analysis of the equilibrium and thermodynamic parameters in terms of the association mechanism demonstrated the involvement of classical hydrophobic interactions in the stabilization of complexes of di- and higher alkylated uracils. Data for the derivatives substituted with polar groups proved consistent with the predominant involvement of dipole-induced dipole forces in the association.

1. Introduction

The vertical self-association of nucleic acid bases and their nucleoside and nucleotide derivatives in aqueous solutions has attracted much attention over the past years [1–8], because of the important contribution of stacking interactions between adjacent base pairs to the free energy of helical polynucleotide structures [9, 10]. In spite of many accumulated equilibrium and thermodynamic data, the nature of these interactions and, in particular, the key role played by water in sustaining the stacked complexes remain unclear. Recently Lawaczeck and Wagner [11] have presented an interpretation of the specificity of the stacking association in terms of dipole-induced dipole interactions, in which the polarizability of the π-electron heterocyclic ring system and the polarizing power of polar bonds were mainly emphasized. The unique solvent contribution to stabilization of stacked complexes in aqueous solutions is considered [12] to be due to the high surface tension of water, because upon stacking the solvent surface area around the solute species decreases. The contribution of the classical hydrophobic interactions, if any, is believed to remain "hidden" [3, 13], in view of: (1) the positive entropy changes accompanying the transfer of nucleic acid bases from organic solvents to water [14], and (2) the negative enthalpies and negative but more variable entropies of the stacking association, derived from van 't Hoff plots [4–7, 15–18] and obtained directly from calorimetric measurements [2, 19, 20] of several systems. However, most of the systems studied so far concerned highly polarizable purine derivatives. Thus, natural diketo-pyrimidines, owing to their lower polarizability, seem to be more suitable for further studies on the relative contribution of hydrophobic and polarization interactions to the stacking association. Involvement of this type of association has been demonstrated [21, 22] in studies on the concentration dependence of photodimerization of several methylated diketo-pyrimidines.

2. Materials and methods

The compounds were obtained by known methods [23] and thoroughly purified. Their purity was checked by melting point determinations and chromatography in several solvent systems.

Measurements of osmotic coefficients at various

solute concentrations were taken according to known procedure [15, 24], using Knauer's (West-Berlin) vapour pressure osmometer. The osmometer was calibrated against sucrose, sodium chloride and d-mannitol used as standards. All substances yielded equivalent calibration plots.

Series of solutions in redistilled water were diluted by weight from stock solutions. The latter were also made up by weight, using dried samples of the substances studied.

The concentration ranges measured were dependent on the sensitivity of the osmometer and solubility of the compounds; they varied from about 0.03 M in the case of the least soluble derivatives (monomethylated and substituted with polar groups) up to about 1 M for di-, tri- and tetra-alkylated uracils.

The mean deviation of the osmotic coefficient at concentrations lower than 0.05 M exceeded 10%, and decreased with rise in concentration of the solute to attain about 2% for 1 M solutions. The most accurate data were obtained for readily soluble substances, usually studied within a wide concentration range.

Since the pK_a values of the substances studied ($4.5 > pK_a > 9.3$) were sufficiently far removed from the pH values of their aqueous solutions, the expected fluctuations of pH near 7 exerted no effect on the stacking equilibria. The abbreviations of the names of compounds used throughout the text conform to the recommendations of the IUPAC-IUB Committee on Biochemical Nomenclature [Biochem. J. 120 (1970) 449] and are explained in tables 1 and 2.

3. Analysis of osmometric data in terms of molecular associations

Analysis of vapour-pressure osmometric data of small molecule aggregating systems is usually performed on two basic assumptions: involvement of a multi-step reversible association of solute and ideal behaviour of all the solute species. According to the Gibbs–Duhem relationship, the molal activity coefficient of a solute γ is related to the osmotic coefficient ϕ of solution by the following equation:

$$\ln \gamma = (\phi - 1) + \int_0^m (\phi - 1)\, d \ln m . \qquad (1)$$

It was also shown [25] that:

$$\ln(m_1/m) = (\phi - 1) + \int_0^m (\phi - 1)\, d \ln m \qquad (2)$$

where m_1 – concentration of the monomer, and m – stoichiometric molal concentration of the solute.

From these relations one can recognize the obvious result that $\gamma = m_1/m$. This permits computation of the monomer concentration m_1 as a function of the molal concentration m of the solute. Then, the stoichiometric concentration m can be expressed by the following series of expansions in which the only unknowns are the consecutive equilibrium constants:

$$m/m_1 - 1 = 2K_2 m_2 + 3K_2 K_3 m_1^2 + \ldots . \qquad (3)$$

Least-squares analysis or linear programming may be applied to determine the coefficients of the polynomial and hence the equilibrium constants [26]. By means of the F-test the best fit is sought with a polynomial of lowest degree, corresponding to the highest aggregating species of significant concentration for an assumed probability level [26].

The unique and physically meaningful solution can be obtained when the accuracy of experimental data is sufficiently high. This is usually not the case and one has to make certain assumptions as to the relative magnitude of the equilibrium constants:

(1) The equilibrium constant for the first step of association $K_2 = \beta K$ is assumed to be different from all the other equilibrium constants considered to be identical: $K_3 = K_4 = \ldots = K_n = K$. Thus eq. (3) can be rearranged to the form:

$$m = m_1 - \beta m_1 + \beta m_1 (1 - K m_1)^2 \qquad (4)$$

and solved for K and β.

(2) All the equilibrium constants are considered to be identical (isodesmic model of association) and can be computed directly from the experimental data [24] from the following expression relating stoichiometric molality and the osmotic coefficient:

$$1 - \phi = K m \phi^2 . \qquad (5)$$

With the aim of comparison of the association tendencies in the whole group of pyrimidine derivatives studied, an analysis of the osmometric data in terms of the above three methods was attempted.

The fitting of eq. (3) and of the F-test (significance

level 0.05) to our data by means of least squares analysis resulted in various association schemes for closely related compounds, what seems to be physically unsound. In many cases the negative coefficients in eq. (3) occurred, what suggested that the varying magnitude of experimental errors was probably responsible for the inadequacy of this most general method of analysis. This method failed with regard to a group of the least soluble monomethylated derivatives and derivatives substituted with polar groups because in these cases representation of ϕ as a polynomial in m is impossible.

Also application of the second, simplified method failed to permit the uniform description of the stacking tendencies, because the parameter β varied from compound to compound and from temperature to temperature, attaining values of $\beta < 1$, $\beta \approx 1$ or $\beta > 1$. Only the isodesmic model described all the data equally well and permitted taking into consideration the measurements of the least soluble monomethylated derivatives and derivatives substituted with polar groups. We have used this model and least-squares analysis of eq. (5) to determine equilibrium constants.

4. Results and discussion

In the figs. 1 and 2, as an illustration of the quality of direct experimental data, the measured osmotic coefficients as a function of concentration together with the smoothed polynomial fits are presented for three compounds differing in their association equilibrium constants and thermodynamics of association: $m_2^{1,3}$Ura at 25°C and 35°C; $m_2^{1,3}h_2^{5,6}$Thy and $e_2^{1,3}$Thy at 25°C.

The values of K_{st} at various temperatures computed according to the isodesmic model of self-association, are recorded in tables 1–3 along with the ΔH^0 values obtained from van 't Hoff plots and the derived values $\Delta G^0_{25°C}$ and ΔS^0. In order to provide a basis for better comparison of our data with the results of other osmometric studies, we also determine K_{st} and the thermodynamic parameters for uridine, thymidine and cytidine (table 3). They agree fairly well with those reported by Ts'o [15], Solie and Schellman [27] and Magar and Steiner [17].

Comparison of K_{st}'s at a given temperature for:

(1) variously N- and C-substituted methyl- and ethyl-uracils (table 1).

(2) a series of 1,3-dimethyluracil derivatives substituted at C5 or C6 with polar groups, and

(3) modified at these positions by aza-substitution, or 5=6 double bond reduction (table 2),

clearly demonstrates that the stacking affinity of 2,4-diketopyrimidine can be controlled by chemical modifications to vary by more than one order of magnitude from $K_{st}^{25°} = 0.22\ M^{-1}$ ($m_2^{1,3}h_2^{5,6}$Ura) to $K_{st}^{25°}$ about 6 M^{-1} ($m_2^{1,3}n^6$Ura, extrapolated value from higher temperatures) or probably to a higher $K_{st}^{25°}$ in

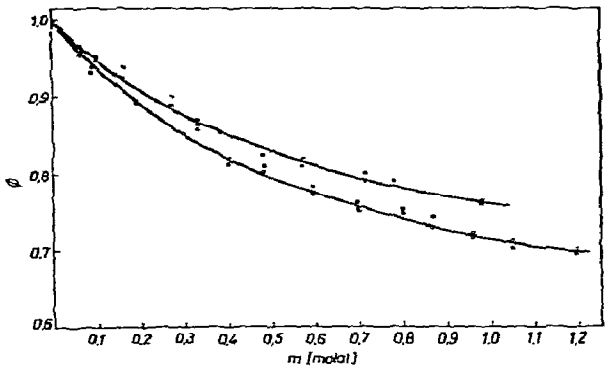

Fig. 1. The osmotic coefficients (ϕ) of $m_2^{1,3}$Ura vs molal concentrations at 25°C (o) and 35°C (•); solid line: smoothed polynomial fit.

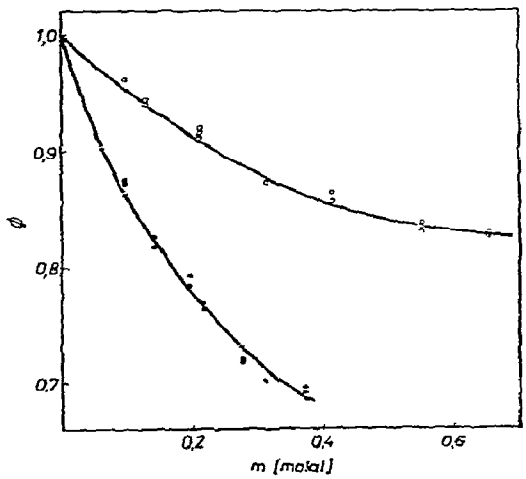

Fig. 2. The osmotic coefficients (ϕ) of $m_2^{1,3}h_2^{5,6}$Thy (o) and of $e_2^{1,3}$Thy (•) vs molal concentrations at 25°C; solid line: smoothed polynomial fit.

Table 1
Equilibrium constants (K_{st}) and thermodynamic parameters of stacking association of alkylated uracils

Compound	K_{st} (molal^{-1})					ΔG^0_{25}° (kcal/mole)	ΔH^0 (kcal/mole)	ΔS^0 (eu)
	25°C	35°C	45°C	60°C	80°C			
1-methyluracil (m^1Ura)	(0.83) [a]	0.6 ± 0.2	0.5 ± 0.1	0.3 ± 0.1		+0.104	−5.2 ± 0.9	−17.8 ± 4.7
3-methyluracil (m^3Ura)	(1.13) [a]	0.73 ± 0.07	0.70 ± 0.10	0.40 ± 0.05		−0.072	−4.9 ± 1.0	−16.2 ± 9.3
1-methylthymine (m^1Thy)	(0.93) [a]	0.7 ± 0.3	0.5 ± 0.2	0.4 ± 0.2		+0.042	−4.1 ± 0.9	−13.9 ± 4.0
1,3,6-trimethyluracil ($m_3^{1,3,6}$Ura)	0.99 ± 0.07	0.97 ± 0.07	0.88 ± 0.07	0.82 ± 0.03		+0.006	−1.1 ± 0.2	−3.8 ± 1.1
3-methylthymine (m^3Thy)	(1.33) [a]	1.3 ± 0.3	1.2 ± 0.2	1.1 ± 0.1		−0.166	−1.0 ± 0.3	−2.8 ± 1.6
1,3,5,6-tetramethyluracil ($m_4^{1,3,5,6}$Ura)	2.2 ± 0.1	2.0 ± 0.1	1.9 ± 0.1	1.86 ± 0.08		−0.455	−0.9 ± 0.1	−1.4 ± 0.6
1,3-dimethylthymine ($m_2^{1,3}$Thy)	1.20 ± 0.20	1.22 ± 0.05	1.16 ± 0.08	1.06 ± 0.08		−0.113	−0.8 ± 0.2	−2.2 ± 1.5
1,3-dimethyluracil ($m_2^{1,3}$Ura)	0.56 ± 0.03	0.51 ± 0.02	0.52 ± 0.04	0.49 ± 0.02		+0.343	−0.6 ± 0.2	−3.4 ± 1.5
1,3-diethyluracil ($e_2^{1,3}$Ura)	1.23 ± 0.05	1.19 ± 0.06	1.27 ± 0.07	1.14 ± 0.05	1.2 ± 0.1	−0.122	~0	~0.5
1,3-dimethyl-5-ethyluracil ($m_2^{1,3}e^5$Ura)	1.3 ± 0.5	1.40 ± 0.10	1.46 ± 0.10	1.43 ± 0.10	0.91 ± 0.15	−0.155	+1.1 ± 0.2 [b]	+4.3 ± 0.6 [b]
1,3-diethylthymine ($e_2^{1,3}$Thy)	1.79 ± 0.06	1.93 ± 0.09	2.0 ± 0.1	2.3 ± 0.1		−0.344	+1.4 ± 0.2	+5.7 ± 1.1

[a] Extrapolated values; [b] obtained from van 't Hoff plot at 25°–45°C.

Table 2
Equilibrium constants (K_{st}) and thermodynamic parameters of stacking association of 5- and 6-substituted 1,3-dimethyluracil derivatives

1,3-dimethyl derivative of	K_{st} (molal^{-1})				$\Delta G^0_{25°}$ (kcal/mole)	ΔH^0 (kcal/mole)	ΔS^0 (eu)
	25°C	35°C	45°C	60°C			
5,6-dihydrouracil ($m_2^{1,3}h_2^{5,6}$Ura)	0.22 ± 0.02				+0.89		
5,6-dihydrothymine ($m_2^{1,3}h_2^{5,6}$Thy)	0.41 ± 0.03				+0.52		
6-azauracil	0.3 ± 0.1				+0.64		
5-azauracil	0.35 ± 0.05				+0.62		
5-hydroxyuracil	0.70 ± 0.08				+0.21		
5-methoxyuracil	1.4 ± 0.2				−0.19		
6-methoxyuracil	1.6 ± 0.1	1.29 ± 0.08	1.28 ± 0.09	1.10 ± 0.05	−0.27	−2.0 ± 0.5	−6.0 ± 3.2
6-aminouracil ($m_2^{1,3}n^6$Ura)	(6.0) [a]	4.0 ± 1.0	2.7 ± 0.8	1.3 ± 0.5	−1.06	−8.0 ± 1.5	−23.5 ± 8.0
5-nitrouracil ($m_2^{1,3}(NO_2)^5$Ura)				2.1 ± 0.3			
5-carbethoxyuracil	(1.23) [a]	1.18 ± 0.05	1.13 ± 0.05	1.08 ± 0.05	−0.12	−0.72 ± 0.06	−2.0 ± 0.3
5-fluorouracil	(1.0) [a]	0.93 ± 0.30	0.77 ± 0.10	0.63 ± 0.10	0.0	−2.3 ± 1.8	−7.7 ± 1.0
5-chlorouracil	1.80 ± 0.10	1.60 ± 0.10		0.85 ± 0.05	−0.38	−4.4 ± 0.7	−13.4 ± 3.7
5-bromouracil				1.6 ± 0.2			
5-iodouracil				1.8 ± 0.2			

[a] Extrapolated values.

Table 3
Equilibrium constants and thermodynamic parameters of stacking association of pyrimidine nucleosides

Compound	K_{st}(molal^{-1})				$\Delta G^0_{25°}$ (kcal/mole)	ΔH^0 (kcal/mole)	ΔS^0 (eu)
	25°C	35°C	45°C	60°C			
uridine	0.64 ± 0.05	0.52 ± 0.04	0,53 ± 0.03	0.44 ± 0.02	0.26	−1.9 ± 0.5	−7.3 ± 3.1
thymidine	1.03 ± 0.09	0.81 ± 0.06	0.74 ± 0.07	0.64 ± 0.05	0	−2.6 ± 0.4	−8.7 ± 1.2
4-thiouridine [a]	2.7 ± 0.2				−0.59		
2-thiouridine [a]	4.5 ± 0.7				−0.89		
2,4-dithiouridine [a]	5.0 ± 0.2				−0.95		
cytidine	0.95 ± 0.08	0.83 ± 0.08	0.77 ± 0.09	0.56 ± 0.04	0.03	−2.9 ± 0.4	−10.0 ± 3.0

[a] From ref. [8].

the case of $m\frac{1}{2}^{,3}(NO_2)^5$Ura and $m\frac{1}{2}^{,3}io^5$Ura. Substitution with both a polar group or an alkyl group generally brings about an increase in K_{st} as it has been noted previously for homoassociation of purines [5, 16, 18, 28] and heteroassociation between purine and pyrimidine derivatives [4, 29].

There is an evident correlation between K_{st} and both electronegativity and polarizability of the substituent in the series of 5-halogeno uracils, in which $K_{st}^{60°}$ increases by a factor of about 3 on going from the fluoro to the iodo derivative (table 2). No such correlation exists, however, between K_{st}'s and the molecular dipole moments of these compounds [30, 31], which are practically independent of the nature of the halogen atom. Stacking affinity also rises with the electron-donating ability of the substituent, as can be seen in the series of 5-substituted $m\frac{1}{2}^{,3}$-uracils (–OH, $-OCH_3$, $-NH_2$). Similarly, $K_{st}^{60°} = 2.1\ M^{-1}$ of $m\frac{1}{2}^{,3}(NO_2)^5$Ura, bearing the strongly electron-accepting nitro group, is about twice as high as $K_{st}^{60°} = 1.1\ M^{-1}$ of the 5-carbethoxy derivative. Substitution, in uridine, of the keto by the thio group of higher polarizability also leads to a distinct increase in K_{st}, as has been shown by us [8], cf. table 3.

On the other hand, reduction of the 5=6 double bond which lowers the π-electron polarizability of the diketopyrimidine ring exerts an opposite effect on its stacking affinity, since $K_{st}^{25°}$ of $m\frac{1}{2}^{,3}h\frac{5,6}{2}$Thy and of $m\frac{1}{2}^{,3}h\frac{5,6}{2}$Ura (table 2) are 2–3 times smaller than those of the respective parent compounds (table 1). To our surprise, 5- and 6-aza substitution had a similar, though lesser, effect.

Investigation of the temperature dependence of K_{st} for a number of uracils bearing polar groups (table 2) showed that their self-association is accompanied by fairly large negative enthalpy and entropy changes. In the case of $m\frac{1}{2}^{,3}n^6$Ura they attain values as high as $\Delta H^0 = -8.1$ kcal mole^{-1} and $\Delta S^0 = -23.5$ eu, being very close to those found for substituted purines [18, 4–7]. With the exception of those for the 5-carbethoxy derivative *, the thermodynamic parameters of association support the conclusion drawn upon analysis of the K_{st} data alone, demonstrating that the stacking ability of this group of compounds is a function of the polarizability of their molecules. Since both keto groups in diketopyrimidines exhibit large dipole bond moments of about 4 debye units [31], the dipole–induced dipole interactions can be invoked to explain the source of stabilization energy of the stacked complexes.

The mechanism underlying self-association of alkyl-uracils seems to be more complicated. Examination of the $K_{st}^{35°}$ data (table 1) shows that the stacking tendency is not a simple function of the number and size of alkyl substituents. The value of K_{st} depends also on the site of substitution (cf. m^1Ura and m^3Ura as well as m^1Thy, m^3Thy and $m\frac{1}{2}^{,3}$Ura). The most striking fact is that the variations in the temperature dependence of K_{st} exhibit negative, close to zero and positive temperature coefficients for closely related compounds, e.g., $e\frac{1}{2}^{,3}$Ura and $e\frac{1}{2}^{,3}$Thy. Consequently, van 't Hoff plots of $\ln K_{st}$ versus T^{-1}(K) yield apparent enthalpies ΔH^0 of association ranging from negative to positive values (fig. 3), and standard entropies ΔS^0, derived there of bear the same sign (table 1). In the case of $m\frac{1}{2}^{,3}e^5$Ura the van 't Hoff

* In the light of the subsequent discussion, the thermodynamics of association of this compound seems to be affected by the hydrophobic interaction between ethyl groups.

plot could not be fitted to a linear function, and exhibits an upward curvature with a flat maximum near 50–60°C (fig. 3). Evidently, the enthalpy of association varies with temperature. Linear approximation of the plot within the higher temperature range corresponding to 60–80°C yields a ΔH^0 value of about -5 kcal mole^{-1}, while within the range of 25–45°C the slope of the initial part of the plot gives a positive enthalpy ΔH^0 of about +1 kcal mole^{-1}.

Thus, it becomes obvious that in most cases the enthalpies obtained from the slopes of the apparently linear van 't Hoff plots could be interpreted as being made up of two contributions with opposite signs corresponding to different physical processes involved in the stabilization of the stacked complexes. The lack of dependence of osmometric K_{st} on temperature for several compounds and its increase with rise of temperature could only be explained under the assumption that base association is partially driven by large positive entropy changes connected with the endothermic process accompanying stacking in solution. This is highly reminiscent of the "hydrophobic bond" formation between hydrocarbon molecules and hydrocarbon amino acid side chains in proteins [32–34], involving contacts between nonpolar groups, with a concomitant decrease in the structural order of water around the solute molecules. Removal of water molecules from intimate contact with nonpolar groups upon their aggregation requires energy to break the hydrogen-bonded water structure around them, with lower entropy than the bulk of the solvent. The thermodynamics of this process is thus characterized by low positive enthalpy and high entropy changes resulting in negative free energies of aggregation, which attain lowest values at about 65°C. Therefore, there is no doubt that the long sought for [3, 12–14, 18, 35] hydrophobic contribution to the stacking association of nucleobases is manifested almost classically [32, 33] in self-association of alkyl uracils.

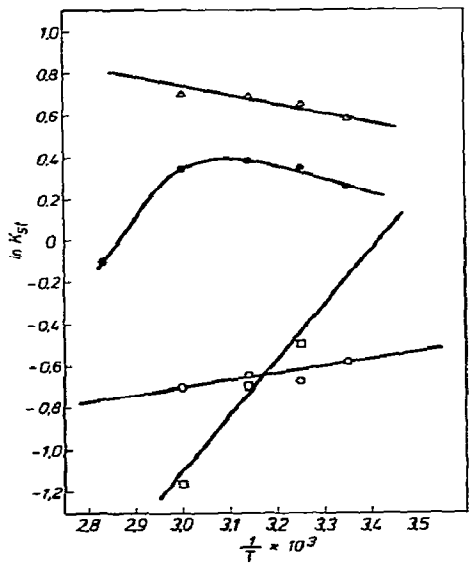

Fig. 3. Van 't Hoff plots of stacking equilibrium constants: (-○-○-○-) $m_2^{1,3}$Ura: (-□-□-□-) m^1Ura: (-△-△-△-) $e_2^{1,3}$Thy: (-●-●-●-) $m_2^{1,3}e^5$Ura.

The fact that the strength of hydrophobic bonding depends primarily on the number of water molecules removed from contact with nonpolar groups explains why the largest hydrophobic contribution to stabilization of the stacked complexes is observed in the solution of highly alkylated uracils and particularly of those bearing ethyl groups. In order to explain the differences in the magnitude of this contribution between variously mono-, di- and tri-methyl substituted uracils, we estimated the number of possible contacts between substituents in the respective stacked homo-dimers, using their CPK models. In view of the demonstrated involvement of electrostatic interactions of the dipole–induced dipole type in the stabilization of the stacked complexes, we considered six possible mutual orientations of the two molecules, in which one carbonyl group of each molecule is situated above the ring of the adjacent molecule. These orientations may be expected to correspond to maximum stabilization energy of electrostatic interactions. They were found for a number of diketopyrimidine crystals and for their crystalline complexes with purines [36]. Only contacts between the alkyl groups themselves were taken into consideration because of the small size of the pyrimidine C–H groups, their interactions with alkyl substituents are not likely to result in substantial hydrophobic bonding. This last point is supported nicely in the case of m^1Ura and m^3Ura (table 1) by the thermodynamic parameters rather typical of electrostatically driven stacking associations. In the case of m^1Ura, in only one of the six rotational isomers of stacked dimers the N1 methyl groups are in contact. For m^3Ura and m^1Thy already three rotational

isomers with single contact are possible while for $m_2^{1,3}$Ura the total number of $CH_3\cdots CH_3$ contacts rises to 7 in five rotational isomers, and to 8 in five isomers in the case of m^3Thy. In tri-methyl substituted $m_2^{1,3}$Thy in each of the six isomers there are two $CH_3\cdots CH_3$ contacts. Because of the expected relatively small differences in the free energies of stabilization between various rotational stacked isomers, they all are likely to be present in solution, and there is a fast rotational exchange between different species during their lifetime of the order of 10^{-9}–10^{-10} s [18, 37].

In the light of the above consideration the classical hydrophobic contribution is expected to be least for the association of m^1Ura, and next in the case of m^1Thy and m^3Ura. This expectation is fully borne out by the experimental K_{st} and thermodynamic data, which conform rather to a model of electrostatic stabilization. With further increase in the probability of the occurrence of hydrophobic contacts between the side methyl groups in $m_2^{1,3}$Ura, m^3Thy and $m_2^{1,3}$Thy, there is a drastic change in the thermodynamics of association. However, introduction of an additional methyl group into $m_2^{1,3}$Thy to form $m_4^{1,3,5,6}$Ura results in but little change in the ΔH^0 and ΔS^0 of association. At the same time, $K_{st}^{25°}$ significantly increases from 1.2 M^{-1} to 2.2 M^{-1}; this might be explained by a rise of the free energy of association, resulting from the somewhat increased polarizability of the molecule upon methyl substitution. The upper limit of this rise, which can be approximately estimated from the difference between the free energies of the m^1Ura and m^1Thy association, is only about one fifth of that between $m_2^{1,3}$Thy and $m_4^{1,3,5,6}$Ura.

We are therefore tempted to explain the increase of K_{st} of $m_2^{1,3}$Thy on substitution with the C6 methyl group as due to the possibility of occurrence in $m_4^{1,3,5,6}$Ura of a greater variety of hydrophobically aggregated species with maximum number of hydrophobic contacts.

The total standard free energy of association can be written as the sum of the electrostatic ΔG_{el} and the hydrophobic ΔG_{hph} contribution:

$$\Delta G^0 = \Delta G_{el} + \Delta G_{hph}$$

$$= \Delta H_{el} + \Delta H_{hph} - T(\Delta S_{el} + \Delta S_{hph}). \qquad (6)$$

The hydrophobic contribution to the free energies of association of all alkyluracils studied can be approximately evaluated when assuming that (1) the thermodynamic parameters of the m^1Ura association reflect mainly the electrostatic contributions to the change in the state of solute upon association and the concomitant change in water structure, and (2) these parameters remain constant for the whole homologous series of alkyluracils. In the light of experimental data and the preceding discussion, the first assumption seems to be well justified. The validity of the second assumption should hold good provided that the distribution of the solute molecules within aggregates with all possible mutual orientations would not vary with the site and number of alkyl substituents and that the introduction of an alkyl group would not affect the state of hydration of the polar carbonyl and N–H groups. However, this does not seem quite probable. One would rather expect a decrease in the electrostatic contribution with the number of alkyl groups. Namely, in stacks hydrophobic interactions should favour molecular packing with $CH_3\cdots CH_3$ overlap, and the adherence of water molecules to sites adjacent to both amide groups [38] should certainly be less favoured upon *N*-alkyl substitution. Consequently, the hydrophobic contributions to the thermodynamic parameters of association (table 4), evaluated on the basis of eq. (6) and of both above mentioned assumptions, are overestimated to an extent dependent on the actual structure of a given alkyl derivative of m^1Ura.

It is of interest to compare some of these values with the standard enthalpies and entropies of formation of hydrophobic bonds between hydrocarbon side-chains in proteins, computed by Nemethy and Scheraga [34] on the basis of their statistical thermodynamic theory of aqueous hydrocarbon solutions [33]. The thermodynamic parameters of formation of the hydrophobic bond between two alanine groups have been predicted to remain within the range of: ΔH^0_{hph} from +0.4 to +0.7 kcal $mole^{-1}$ and ΔS^0_{hph} from +2.1 to +4.7 eu, depending on the actual geometry of adherence of both groups. In the case of the $m_2^{1,3}$Thy association we arrived at $\Delta H^0_{hph} = 4.4$ kcal $mole^{-1}$ and $\Delta S^0_{hph} = 15.6$ eu; formally this would correspond to the formation of two alanine–alanine hydrophobic bonds, because in each of the six lowest-energy rotational isomers of stacked $m_2^{1,3}$Thy dimers there are two pairs of $CH_3\cdots CH_3$ contacts. Bearing in mind that our data are rather overestimated and that they comprise also the hydrophobic contri-

Table 4
Calculated [eq. (4)] enthalpies and entropies of "hydrophobic bonding" in stacked complexes of alkylated uracils

Compound	ΔH^0_{hph} (kcal mole^{-1})	ΔS^0_{hph} (eu)
m^3Ura	0.3	1.6
m^1Thy	1.1	3.9
$m_3^{1,3,6}$Ura	4.1	14.0
m^3Thy	4.2	15.0
$m_3^{1,3,5,6}$Ura	4.3	16.4
$m_2^{1,3}$Thy	4.4	15.6
$m_2^{1,3}$Ura	4.6	14.4
$e_2^{1,3}$Ura	5.2	17.3
$m_2^{1,3}e^5$Ura	6.3	22.1
$e_2^{1,3}$Thy	6.6	23.5

bution from higher aggregates, the two sets of thermodynamic data exhibit fairly good agreement as to the order of magnitude.

An attempt was also made to analyse the temperature-dependence of the thermodynamic parameters of hydrophobic bonding in terms of the Nemethy–Scheraga theory [34] in the case of the $m_2^{1,3}e^5$Ura association. In view of the distinct curvature of $\ln K_{st}$ vs T^{-1}, according to this theory, ΔG^0_{hph} can be expressed in the form of a second degree function of temperature:

$$\Delta G^0_{hph} = a + bT + cT^2 \qquad (7)$$

and the remaining thermodynamic parameters derived thereof are the following functions of temperature and of the empirical parameters a, b and c:

$$\Delta H^0_{hph} = a - cT^2 , \qquad (8)$$

$$\Delta S^0_{hph} = -b - 2cT, \qquad (9)$$

$$(\Delta c_p)_{hph} = -2cT \qquad (10)$$

Under the assuption that the electrostatic contribution to enthalpy of association is independent of temperature, this being justified as a first order approximation because of the apparent linearity of the $\ln K_{st}$ vs T^{-1} plot in the case of m^1Ura, we calculated $\Delta G^T_{hph} = \Delta G^T(m_2^{1,3}e^5\text{Ura}) - \Delta G^T(m^1\text{Ura})$ at various temperatures using the experimental K^T_{st} and fitted the so obtained ΔG^0_{hph} values to eq. (7). The results are shown in fig. 4, in which the solid line represents the computed function with parameters $a = 31088$, $b = -182$, $c = 0.25$ and the squares the experimental ΔG^0_{hph} values. The fit is very good indeed, with a mean deviation of 6%. At 25°C the thermodynamic functions attain the values: $\Delta H^0_{hph} = 8.8$ kcal mole^{-1} and $\Delta S^0_{hph} = 33$ eu, while $(\Delta c_p)_{hph}$ amounts to -150 kcal mole^{-1} deg^{-1}. They are by about 50% higher than those obtained by direct subtraction of the respective $\Delta G^{25°}$ of $m_2^{1,3}e^5$Ura and m^1Ura (table 4). Taking into account the average (3) number of hydrophobic contacts between the alkyl groups in $m_2^{1,3}e^5$Ura lowest-energy stacked dimers, we come once again to the conclusion that there is fair agreement between the present and the predicted [34] values of the thermodynamic parameters of hydrophobic interactions between hydrocarbon side-chains. The change in the heat capacity of formation of the hydrophobic bond between alkyluracils per side-chain pair $(\Delta c_p)_{hph}$ -50 kcal mole^{-1} deg^{-1} also remains within the range of the predicted values (-10 to -50 kcal mole^{-1}deg^{-1}).

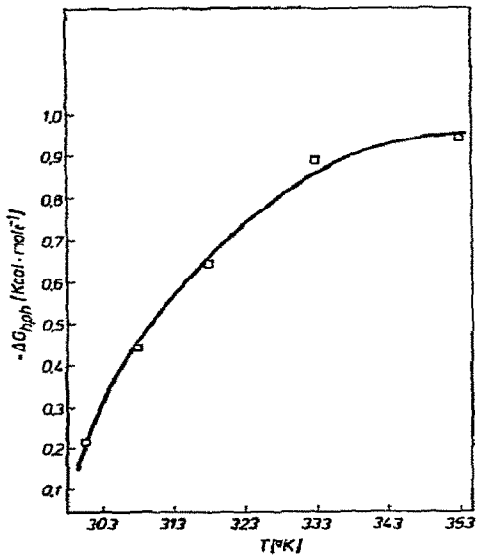

Fig. 4. Temperature dependence of hydrophobic contribution to free energy G^0_{hph} of stacking association for $m_2^{1,3}e^5$Ura: (○) calculated values ($\Delta G^0_{hph} = \Delta G^0 - \Delta G^0_{m^1\text{Ura}}$); solid line: fit to the function $\Delta G^0_{hph} = 31088 - 182T + 0.25T^2$.

If the hydrophobic contribution to the free enthalpy of stacking ΔG^0 is a second-order function of the temperature, the question arises, why in most our experiments the $\ln K_{st}$ vs T^{-1} plots are apparently linear? There is no doubt that the low precision of osmometric determinations of the association constants, as well as the limited range of temperature changes, can be the main reason of our inability to distinguish between linear and nonlinear fits. Consequently, the results obtained for $m_2^{1,3}e^5$Ura might be rather fortuitous. However, other factors could also be responsible for the observed slopes of the plots.

First of all, since each "hydrophobic bond" is characterized by a specific temperature of its maximum stability, in an aggregating system bearing several alkyl groups on the monomeric units one would expect their continuous redistribution between various possible aggregates with rising temperature. This expectation finds strong support in the result of our recent photochemical studies on photodimerization of some alkylated diketopyrimidines in concentrated aqueous solutions [21, 22].

Under these conditions photodimerization is almost exclusively a very fast excited singlet state reaction ($K_{\text{dim. form.}} \geqslant 10^{12}\ s^{-1}$) taking place within association complexes formed prior to excitation. Thus, complexes with mutual orientation of monomeric units appropriate for cyclobutane-type dimer formation, become photoselected and their relative content is manifested in the distribution of the dimeric photoproduct between various steroisomers of the cylcobutane dimer. This distribution is different for each studied compound and varies with temperature in a characteristic manner. For instance, in the case of $m_2^{1,3}$Thy in 0.02 M solution at 26°C only two *cis* stereoisomers (*syn* and *anti*) were formed with an almost equal probability, whereas at higher temperatures the third *trans–anti* dimer appeared in an increasing proportion at the cost of the two former species. The apparent enthalpy of dissociation of the precursor ground state complex of the latter dimer varied with temperature from positive to negative values of about 1 kcal mole^{-1} within the temperature range of 25–75°C, as it could be inferred from the van 't Hoff plot $\ln \phi_{df}$ vs T^{-1}. Thus, the *trans–anti* isomer is probably formed from some laterally aggregated species which correspond to maximum hydrophobic stabilization. On the contrary, $\ln \phi_{df}$ vs T^{-1} plots for both *cis* isomers yielded negative enthalpies of association of the respective precursor complexes: an apparently temperature-independent enthalpy of about 4.5 kcal mole^{-1} in the case of the *cis–syn* dimer, and a temperature-dependent one which took on progressively less negative values from about −8 to about −2 kcal mole^{-1} in the case of the *cis–anti* dimer. The average enthalpy of photoselectable complexes $\Delta H^0 = -2.4 \pm 0.2$ kcal mole^{-1} [21] is much more negative than that derived from osmometric data (table 1). In other terms, within the temperature range studied, the quantum yield of dimer formation (ϕ_{df}) from photoselectable complexes, and hence also the concentration of the latter, is reduced to about one half of the initial value at 25°C, whereas at the same time the total concentration of aggregates drops by no more than about 20%. This clearly indicates that, besides the photoselectable complexes with high electrostatic contribution to their stabilization, a number of other complexes – stabilized to a high extent hydrophobically – occur in solution. Some of these complexes differ in their maximum temperature stability.

In the light of the above considerations there remains little doubt that the isodesmic model of association oversimplifies the actual processes of stacking interactions which exhibit far greater specificity and diversity than it is tacitly assumed in the model. In fact, for each polymerization step several separate equilibrium constants should be considered and, moreover, their variation with the polymerization number and actual packing mode could to some extent be expected. Unfortunately, the osmometric data alone neither provide sufficient information nor are accurate enough to permit deeper insight into the molecular mechanism of association with the aid of such complicated models.

Finally, consideration has to be given to the specific hydration of polar groups within stacked complexes, being likely to contribute also to their stabilization beside interfacial energy [12]. Recent theoretical calculations [38] have demonstrated the occurrence, in the first hydration layer of thymine, of two favourable, symmetrically situated hydration sites on each side of the C(2)=O(2) carbonyl oxygen and a third one between N(3)–H and the C(4)=O(4) carbonyl oxygen. Bonding energies in all three sites were found to be similar for both coplanar and perpendicular

orientations of H_2O with respect to the plane of the thymine ring. These findings, as well as similar results obtained for other nucleobases indicate that the most stable position of water already hydrogen-bonded to one atom is generally determined by another neighbouring proton-donor or proton-acceptor atom [39]. If so, then for certain mutual orientations of monomers in stacked complexes, corresponding to maximum electrostatic stabilization, vertical bridging of polar groups by H_2O molecules may be a more favourable mode of hydration, and hence an additional source of the driving force of the stacking association. The varying degree of hydration with temperature can thus be another factor contributing to the apparent constancy of the enthalpy of association in cases of simultaneous electrostatic and hydrophobic stabilization of complexes; namely, the respective discrete contributions to enthalpy are expected to be similar, though opposite, functions of temperature.

In cases of predominant electrostatic and specific hydration stabilization, a progressive decrease in the enthalpy of complex formation with increase of temperature could be expected. Provided that the experimental data are sufficiently accurate, one would then observe the temperature dependence of the free energy of association to be a second-order function with downward curvature opposite to that of hydrophobically stabilized aggregates.

It is obvious that much more experimental and theoretical work must be done in order to verify the proposed modes of association and the underlying mechanisms. Direct microcalorimetric determination of enthalpies of association, heats of hydration and heat capacities at various temperatures and concentrations of solutes should be particularly useful in this respect.

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