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Stacking Interactions of Nucleobases: NMR-Investigations
II. Selfassociation of Purine- and Pyrimidine-Derivatives

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Abstract. The selfassociation of various purine- and pyrimidine-derivatives in D_2O has been studied by means of NMR technique. The thermodynamic quantities have been calculated using an isodesmic NMR model.

Among the nucleobases investigated, the adenine-derivatives were found to be most suitable for quantitative determination. A comparison of methylated adenine-derivatives and the *pH*-dependence of the selfassociation lead to the conclusion, that the stacking associates are stabilized by special van der Waals interactions based, essentially, on the polarizability of the π-electron-system of the associated molecules.

Key words: Stacking — Selfassociation — NMR — Nucleobases.

Introduction

The stacking selfassociation of nucleobases in aqueous solutions has not yet been elucidated. Apparently, the solvent water is the "driving force" and would be, therefore, an indispensable condition for stacking interaction (Ts'o, 1970; Pörschke and Eggers, 1972). The complexes, however, will be stabilized most probably by special van der Waals forces (Ts'o, 1970; Lawaczeck and Wagner, 1974).

In order to compare stacking associates, several thermodynamic quantities have to be determined. Various experimental techniques have been used in order to obtain these thermodynamic quantities, for instance vapor pressure osmometry (Pörschke and Eggers, 1972), microcalorimetry (Marenchic and Sturtevant, 1973), and NMR spectroscopy as well.

In the nuclear magnetic resonance (NMR) studies conducted (Broom *et al.*, 1967; Lawaczeck, 1972; Antonovsky *et al.*, 1973; Dimicoli and Helene, 1973), different models and methods of calculation have been applied. As has been pointed out previously (Schimmack *et al.*, 1975) the isodesmic NMR model of Dimicoli and Helene (1973) seems to be quite appropriate for the calculation of thermodynamic quantities of stacking associates. In the present study, this model is applied to the selfassociation of some derivatives of nucleobases in aqueous solutions. The results are compared with those obtained by other authors using different experimental techniques and are discussed in regard to the current hypothesis of stacking interaction.

Materials and Methods

The purine- and pyrimidine-derivatives used in this study are abbreviated as "nucleobases" or, simply, as "bases". N^9-ethyladenine (e^9Ade), N^6,N^9-dimethyladenine (m^6m^9Ade), N^6-dimethyl-N^9-ethyladenine ($m_2^6e^9$Ade), N^6-dimethyladenine (m_2^6Ade), and N^9-ethylhypoxanthine (e^9Hyp) have been purchased from Cyclo Chemical Company, Los Angeles, USA. Coffein, tetramethyl-uric-acid (TUA), N^1-methylcytosine (m^1Cyt), and N^1,N^3-dimethyluracil (m^1m^3Ura) were obtained from Fluka, Buchs, Switzerland. N^6-dimethyladenosine (m_2^6Ado), D_2O, the buffer components, and tertiary butanol (tB) were purchased from Merck-Schuchard, Darmstadt, Germany.

The nucleobases have been used without further purification with the exception of coffein, which has been recristallized from H_2O.

All spectra have been recorded on a Varian HA-100 spectrometer equipped with a variable temperature system. The temperatures have been calculated according to a calibration proposed by Van Geet (1968 and 1970).

All chemical shifts in D_2O were measured relative to tB used as an internal standard. The investigations concerning the *pH*-dependency were performed without using tB because of its ability to react with some buffer components. In this case the chemical shifts were measured relative to the H_2O-lock and, then, referred to shifts relative to tB measured in a separate probe.

All calculations have been performed on a Wang computer 600-14.

The thermodynamic quantities, the apparent equilibrium constants K (at 25 C: K^{25C}), the enthalpies ΔH, and the entropies ΔS have been calculated according to the isodesmic NMR model described recently (Schimmack *et al.*, 1975). This model has three NMR parameters: the monomer shift δ_M, the dimer shift Δ_2, and the trimer shift Δ_3 (both measured relative to δ_M). The calculations have been simplified by choosing $\Delta_3 = 2 \cdot \Delta_2$ that is by selecting the special model proposed by Dimicoli and Helene (1973).

In general, Δ_2 proved to be temperature dependent and was approximated by linear functions:

$$\Delta_2(T) = \Delta_2^{0C} + m_\Delta \cdot T$$

(T: temperature in C; m_Δ: temperature coefficient).

The average errors of the thermodynamic quantities of a certain proton of a substance investigated are ± 15% for ΔH and ± 20% for K and ΔS as well. If the values obtained for the different protons of the same molecule are averaged ("proton-averaged values"), the errors are less than ± 10% for ΔH and ± 15% for ΔS, resp.

Results

The proton magnetic resonances of all nucleobases investigated were shifted upfield with increasing concentration of the bases (Fig. 1). The upfield shifts of the adenine-derivatives were considerably more pronounced than those of the other bases due to the higher strength of their ring currents (Giessner-Prettre and Pullman, 1965). The adenine-derivatives (Fig. 2), therefore, are very suitable for quantitative evaluations.

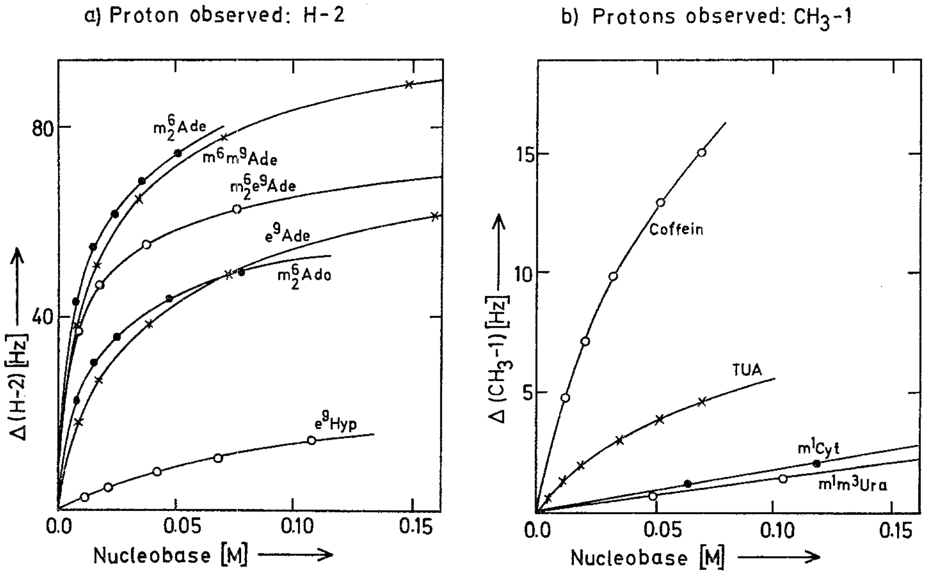
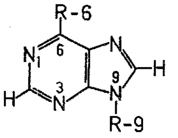


Fig. 1. Selfassociation of various nucleobases in D_2O at 4° C



R-6	R-9	abbreviation
NH_2	CH_2CH_3	e^9Ade
$NHCH_3$	CH_3	m^6m^9Ade
$N(CH_3)_2$	CH_2CH_3	$m^6_2e^9$Ade
$N(CH_3)_2$	H	m^6_2Ade
$N(CH_3)_2$	Ribose	m^6_2Ado

Fig. 2. The adenine-derivatives

From the other derivatives investigated e^9Hyp and coffein only have been choosen for the calculations since the upfield shifts of TUA and the pyrimidine-derivatives appeared to be too small for evaluation.

The results obtained for the selfassociation in D_2O are summarized in Tables 1 to 3. Because of the basic and acidic pK-values of the bases the selfassociation should be pH-dependent. This can be seen in Table 4 in the case of m^6_2Ade ($pK_a = 3.9$; $pK_b = 10.5$) and m^6m^9Ade (no pK_b due to the methylation at N-9).

From the results obtained it can be seen:

1. The enthalpies and entropies calculated from the chemical shifts of different protons of the same nucleobase agree within the experimental errors (Table 1). The absolute values of $K^{25\,C}$ are more dependent on the proton observed than ΔH and ΔS (see also Schimmack *et al.*, 1975).

2. Within the adenine-derivatives the dimer shifts $\Delta_2^{0\,C}$(H-2) of the H-2-protons are always more pronounced than those of the other protons (Table 2). The dimer shifts of the protons H-8 and (not shown in Table 2) R-9 in units of

Table 1. Selfassociation of various nucleobases in D_2O: thermodynamic quantities of the protons observed (R-6 and R-9 see Fig. 2; *pD*-values see Table 3)

Base	Proton											
	H-2			H-8			R-6			R-9		
	K^{25C}	$-\Delta H$	$-\Delta S$	K^{25C}	$-\Delta H$	$-\Delta S$	K^{25C}	$-\Delta H$	$-\Delta S$	K^{25C}	$-\Delta H$	$-\Delta S$
e^9Ade	17.0	4.9	10.9	10.1	6.1	16.0				11.3[a]	4.9	11.7
m^6m^9Ade	32.0	7.3	17.8	21.5	7.7	19.6	25.8	6.5	15.4	21.0	6.6	16.1
$m_2{}^6e^9$Ade	65.4	6.6	13.7	41.8	6.7	14.9	43.2	6.2	13.4	37.1[a]	6.2	13.7
$m_2{}^6$Ade	46.5	9.0	22.6	35.3	9.9	26.1	34.0	9.4	24.5			
$m_2{}^6$Ado	35.9	6.1	13.3	13.1	6.5	16.6	22.8	5.6	12.6	20.8[b]	6.6	16.1
e^9Hyp	3.2	4.6	13.1	2.5	4.4	12.9				1.6[a]	4.1	12.7
	CH_3-1			[c]			CH_3-3			CH_3-7		
Coffein	8.3	3.5	7.6				9.8	4.0	8.8	8.9	3.2	6.4

[a] CH_2 of the ethyl-group
[b] H-1′ of the ribose
[c] not determinable

units: K^{25C} $[M^{-1}]$
ΔH [kcal · mol^{-1}]
ΔS [cal^{-1} · mol^{-1} · degree^{-1}]

Δ_2^{0C}(H-2) are quite similar for these derivatives and vary between 0.5 and 0.7. The dimer shifts of the methyl protons of the ethyl groups are not listed in Table 2, since they are very small ($\lesssim$ 10 Hz) and exhibit considerable errors. Within the adenine-derivatives Δ_2^{0C}(H-2) decreases as follows:

$$m^6m^9\text{Ade} > m_2^6\text{Ade} > e^9\text{Ade} > m_2^6e^9\text{Ade} > m_2^6\text{Ado}.$$

3. The proton-averaged apparent equilibrium constants K^{25C} are always smaller than those ones obtained by other authors (Table 3). This may indicate that the calculations have to be performed with a value of γ (trimer to dimer shift; Schimmack *et al.*, 1975) greater than 2. A comparison of the K^{25C} values of the substances investigated seems to be possible since the sequence in the K^{25C} values can be correlated to the one obtained by other authors:

$$m_2^6e^9\text{Ade} > m_2^6\text{Ade} > m^6m^9\text{Ade} > m_2^6\text{Ado} > e^9\text{Ade} > \text{coffein} > e^9\text{Hyp}.$$

4. The proton-averaged enthalpy values agree very well with the results obtained by the other authors (Table 3). An exception is m^6m^9Ade, of which similar results have been obtained using phosphate buffer (*pD* 7.4) (Schimmack *et al.*, 1975). The sequence of the enthalpy values is:

$$m_2^6\text{Ade} > m^6m^9\text{Ade} > m_2^6e^9\text{Ade} > m_2^6\text{Ado} > e^9\text{Ade} > e^9\text{Hyp} > \text{coffein},$$

a result which differs slightly from the sequence of the K^{25C} values.

5. The selfassociation of the bases is diminished by either deprotonation or — to a larger extent — by protonation (Table 4). Thus, the selfassociations are, in most of the cases, too small for evaluation at *pD*-values less than 4. Because of the *pK*-values near 10 and 4, resp., the selfassociation of the purines exhibits its maximum at neutral *pH*. Purines substituted at the N-9 position are not deprotonable. m^6m^9Ade, *e.g.*, shows, therefore, no decrease in selfassociation with increasing *pD*-value (Table 4).

Table 2. Selfassociation of various nucleobases in D_2O: NMR-parameters of the protons observed

Base	Proton												$\frac{\Delta_2{}^{oc}(\text{H-8})}{\Delta_2{}^{oc}(\text{H-2})}$
	H-2			H-8			R-6			R-9			
	δ_M	$\Delta_2{}^{oc}$	m_Δ	δ_M	$\Delta_2{}^{oc}$	m_Δ	δ_M	$\Delta_2{}^{oc}$	m_Δ	δ_M	$\Delta_2{}^{oc}$	m_Δ	
e^9Ade	8.279	48.5	− 0.37	8.164	32.7	− 0.09				4.273[a]	31.1	− 0.16	0.67
m^6m^9Ade	8.293	59.3	− 0.18	8.022	36.0	+ 0.04	3.127	30.3	− 0.16	3.810	31.3	− 0.07	0.61
$m_2{}^6e^9$Ade	8.217	44.1	− 0.18	8.067	25.2	+ 0.01	3.445	32.4	− 0.07	4.221[a]	24.8	− 0.08	0.57
$m_2{}^6$Ade	8.214	53.3	− 0.07	8.106	37.4	+ 0.14	3.445	39.8	+ 0.16				0.70
$m_2{}^6$Ado	8.237	37.3	− 0.14	8.246	18.4	+ 0.10	3.463	33.8	− 0.03	6.071[b]	18.8	− 0.01	0.49
e^9Hyp	8.205	24.1	− 0.12	8.147	21.7	− 0.05				4.286[a]	29.1	+ 0.01	0.90
	CH_3-1						CH_3-3			CH_3-7			
Coffein	3.363	20.7	+ 0.01	7.875	[c]	[c]	3.546	20.7	+ 0.03	3.964	13.6	0.00	

[a—c] see Table 1 units: δ_M [ppm] $\Delta_2{}^{oc}$ [Hz] m_Δ [Hz · degree^{-1}]

Table 3. Selfassociation of various nucleobases in D_2O: comparison of proton-averaged NMR-values (this study) with values obtained by other authors

Base	This study				Other authors			References
	pD	K^{25C}	$-\Delta H$	$-\Delta S$	K^{25C}	$-\Delta H$	$-\Delta S$	
e^9Ade	7.8	12.5	5.3	12.9				
m^6m^9Ade	7.9	24.7	7.0	17.2	45.3	8.7	21.6	Pörschke and Eggers, 1972
					26.4	8.2	21.0	Antonovsky *et al.*, 1973
$m_2{}^6e^9$Ade	7.9	45.9	6.4	13.9				
$m_2{}^6$Ade	7.9	38.0	9.4	24.4	56.6	9.1	22.5	Marenchic and Sturtevant, 1973
$m_2{}^6$Ado	7.6	21.7	6.2	14.6	33.3	6.4	14.5	Bretz *et al.*, 1974
e^9Hyp	7.1	2.3	4.4	12.9				
Coffein	7.8	9.0	3.6	7.6	15.2	3.4	6.0	Gill *et al.*, 1967

units: K^{25C} $[M^{-1}]$
ΔH $[\text{kcal} \cdot \text{mol}^{-1}]$
ΔS $[\text{cal} \cdot \text{mol}^{-1} \cdot \text{degree}^{-1}]$

Table 4. Selfassociation of $m_2{}^6$Ade and m^6m^9Ade at various *pD*-values (calculations have been done without using an internal reference)

Base	*pD*	Proton									
		H-2		H-8		H-2			H-8		
		δ_M	$\Delta_2{}^{OC}$	δ_M	$\Delta_2{}^{OC}$	K^{25C}	$-\Delta H$	$-\Delta S$	K^{25C}	$-\Delta H$	$-\Delta S$
$m_2{}^6$Ade	13.4	8.089	21.7	7.893	12.8	5.0	8.6	26.9	3.6	6.9	22.0
	10.4	8.194	54.5	8.077	41.3	34.8	9.5	26.3	28.8	8.0	21.7
	7.6	8.201	52.8	8.111	39.1	40.4	10.1	27.9	39.8	9.3	25.4
	5.4	8.242	63.1	8.157	49.9	20.2	6.9	18.5	17.6	5.9	15.6
	3.9	8.261	31.5	8.172	[a]	3.6	3.5	10.5	[a]		
m^6m^9Ade	13.4	8.284	59.7	8.046	38.9	27.7	8.5	23.2	28.2	6.9	18.0
	10.4	8.275	59.5	8.051	39.5	26.2	8.5	23.4	27.5	6.7	17.1
	7.4	8.294	62.8	8.029	39.2	32.7	7.2	17.2	21.1	7.8	20.1
	5.4	8.302	64.4	8.037	40.2	21.4	6.6	15.9	10.4	8.0	22.3
	2.9	8.429	[a]	8.273	41.3	[a]			2.9	2.3	5.6

[a] not determinable units: see Tables 2 and 1

The *pD*-values (*pD*: $= pH + 0.4$) have been adjusted as follows:

pD 13.4:	0.05 m NaOD
pD 10.4 and *pD* 7.6:	Britton-Robinson buffer I
pD 7.4:	phosphate buffer
pD 5.4:	acetate buffer
pD 3.9 and *pD* 2.9:	0.02 m citric acid

Discussion

From the results obtained it is evident, qualitatively, that the selfassociation of the adenine-derivatives is more pronounced than in the cases of either the other purine-derivatives or the pyrimidine-derivatives. The selfassociation of nucleobases, therefore, decreases as follows:

$$\text{Ade} > \text{Hyp} > \text{Cyt} \gtrsim \text{Ura}.$$

The total dipole moments, the total polarizabilities, or the ionization potentials don't seem to be the determining factors of the selfassociation of the nucleobases as has already been pointed out by other authors (Ts'o, 1970; Lawaczeck and Wagner, 1974). It may be noted, however, that the resonance energy of the bases calculated by Pullman and Pullman (1963) agrees very well with the intensity of the selfassociations observed.

Since the resonance energy is a measure of the delocalization of the π-electron-system (delocalization energy), it may be correlated to the polarizability of the π-electron-system. Then, the correlation between thermodynamic quantities and delocalization energy would be consistent with the current hypothesis of stacking interaction. Ts'o (1970), *e.g.*, assumes that the π-electron-polarizability is involved in the stabilization of stacking associates. Lawaczeck and Wagner (1974) assume that the dipole-induced dipole interaction due to the polarizing effectiveness of the substituents and the polarizability of the aromatic ring systems is fundamental for stacking interactions.

As can be seen from the *pD*-dependence of the monomer shift of the ring protons H-2 and H-8 in the cases of m_2^6Ade and m^6m^9Ade (Table 4) the shielding of the whole ring system is influenced by protonation or deprotonation. Therefore, it may be suggested that protonation as well as deprotonation influences also the polarizability of the π-electron-system. Deprotonation enhances the delocalization energy and, therefore, may enhance the polarizability, too. On the other hand, protonation may diminish the polarizability. Thus, the stacking selfassociation of the completely deprotonated bases will be impeded due to the repulsion of the negative charges, yet it is not impossible as has been observed in the cases of completely protonated bases.

Moreover, the negative charges of purines deprotonated at N-9 can be separated enough to allow, for instance, an antiparallel stacking. In the case of protonation which is not limited to a definite side of a molecule such an appropriate arrangement seems not always to be possible. In this case, the average overlapping of aromatic systems of selfassociating molecules is diminished and, therefore, the van der Waals interaction may be reduced.

The thermodynamic quantities obtained for the adenine-derivatives also support the hypothesis that a stabilization by van der Waals forces is important for the stacking effect. Among the adenine-derivatives investigated m_2^6Ade exhibits the greatest value of $-\Delta H$ and $m_2^6e^9$Ade the one of $K^{25\,C}$. The latter agrees with the well established rule that alkylation, especially methylation, enhances the tendency of association (Pörschke and Eggers, 1972; Helmkamp and Kondo, 1968): $m_2^6e^9$Ade is the most alkylated compound.

As can be seen from the results obtained for e^9Ade and $m_2^6e^9$Ade methylation enlarges the enthalpy values. Obviously, additional factors might influence the selfassociation tendency since the enthalpy values decrease in the following order:

$$m_2^6\text{Ade} > m^6m^9\text{Ade} > m_2^6e^9\text{Ade} > m_2^6\text{Ado}\,.$$

In regard to the hydrogen atom in the five membered ring of the adenine-derivatives a substitution at N-9 might interfere with an equivalence of the nitrogen atoms N-7 and N-9, and can, thus, result in a decrease of the polarizability (Ts'o, 1970). Moreover, larger substituents, such as ribose and ethyl groups in the cases of m_2^6Ado and $m_2^6e^9$Ade, resp., can interfere with an overlap of the π-electron-systems of the bases. The dimer shifts of m_2^6Ado or $m_2^6e^9$Ade are less than those ones of m_2^6Ade. This may indicate a somewhat increased distance between the ring planes of the bases. A small increase in that distance can result namely in a considerable decrease in the binding energy caused by the dependence of the van der Waals forces on the distance of the interacting molecules.

Summarizing it may be concluded that the results obtained are best understood by correlating the stabilization of stacking selfassociates to special van der Waals interactions based essentially on the polarizability of the π-electron-system of the bases.

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