Biophys. Struct. Mechanism 1, 113—120 (1975)

Stacking Interactions of Nucleobases: NMR-Investigations
I. Selfassociation of N^6,N^9-Dimethyladenine and N^6-Dimethyl-N^9-Ethyladenine* **

W. Schimmack, H. Sapper, and W. Lohmann

Abteilung für Biophysikalische Chemie, GSF, D-8042 Neuherberg,
and Institut für Biophysik der Universität, D-63 Giessen,
Fed. Rep. Germany

Received July 16, 1974

Abstract. The selfassociation of N^6,N^9-dimethyladenine and N^6-dimethyl-N^9-ethyladenine has been studied by means of NMR technique. The thermodynamic quantities have been calculated using an isodesmic NMR model with three NMR parameters (the monomer shift δ_M and two complex shifts Δ_2 and Δ_3).

The dependence of the thermodynamic quantities on the NMR parameters is discussed. Special attention is given to the determination of δ_M and its temperature dependence.

Calculations with $\Delta_3 = 2 \cdot \Delta_2$ and δ_M taken independently of temperature result in an average entropy $\Delta S = -17.9 \pm 1.8$ e.u. for N^6,N^9-dimethyladenine and $\Delta S = -16.7 \pm 1.7$ e.u. for N^6-dimethyl-N^9-ethyladenine and in an average enthalpy $\Delta H = -7.2 \pm 0.6$ kcal $\cdot$ mol^{-1} for both substances investigated.

Key words: Stacking — selfassociation — NMR — Isodesmic Model — Enthalpy.

Introduction

The selfassociation of nucleobases in aqueous solution represents a special case of the so-called stacking interaction, which has been studied by several authors using different experimental techniques (Antonovsky *et al.*, 1973; Bretz *et al.*, 1974; Dimicoli and Helene, 1973; Lawaczeck, 1972; Marenchic and Sturtevant, 1973; Pörschke and Eggers, 1972; Ts'o, 1970). The determination of the thermodynamic quantities of this interaction requires however, certain simplifications in regard to the thermodynamic model applied and the characteristic parameters of the technique used.

In the nuclear magnetic resonance (NMR) technique these parameters are the chemical shifts of a proton of a monomer (monomer shift) and of the various associates existing in the solution (complex shift).

For the explanation of the selfassociation phenomena the application of the isodesmic model seems to be most appropriate (Bretz *et al.*, 1974; Pörschke and Eggers, 1972). This model is based on the assumption that an infinite number of equilibria exists in the solution which all have the same [or two at the most (Pörschke and Eggers, 1972)] equilibrium constant. The corresponding NMR model exhibits, then, an equivalent number of complex shifts as equilibria exist.

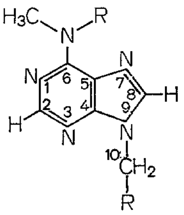
---

* Part of the Ph.D. Theses of W. Schimmack and H. Sapper.

** Dedicated to Professor Dr. A. Schraub on the occasion of his 65th birthday.

Obviously, it is necessary to simplify the model. The most simple assumptions consider the existence of only one chemical shift as average of all possible shifts in the associate (Antonovsky *et al.*, 1973) or two chemical shifts (Dimicoli and Helene, 1973) resulting from the protons of both molecules at the ends of an associate (dimer shift) and from the protons in the other (n-2) molecules of the associate (trimer shift).

In this study, an isodesmic NMR model with two complex shifts is used. The quotient of these shifts is varied since there is no physical reason for keeping it constant. Some of the models proposed previously are special cases of this model. A trimer-to-dimer shift quotient of 2 corresponds to the model applied by Dimicoli and Helene (1973) while a quotient of 1 means the existence of only one chemical shift taken as average of all possible chemical shifts (Antonovsky *et al.*, 1973). The model is tested on the selfassociation of N^6,N^9-dimethyladenine and N^6-dimethyl-N^9-ethyladenine.



R = H: N^6,N^9-dimethyladenine
R = CH_3: N^6-dimethyl-N^9-ethyladenine

The dependence of the thermodynamic quantities on the NMR parameters is discussed. Because of the importance for the evaluation of NMR data of self-association, special attention is given to the determination of the monomer shift and its temperature dependence.

Materials and Methods

N^6,N^9-dimethyladenine (m^6m^9Ade) and N^6-dimethyl-N^9-ethyladenine ($m^6_2e^9$Ade) have been purchased from Cyclo Chemical Company, Los Angeles, USA, and used without further purification. D_2O, the reference substance TPA (2,2,3,3-tetra-deutero-3-(trimethylsilyl)-propionic acid Na-salt), and the buffer components have been purchased from Merck, Darmstadt, Germany.

Eight samples of each, m^6m^9Ade and $m^6_2e^9$Ade, in the concentration range between 0.002 and 0.2 M have been measured relative to the lock H_2O (8%) at pD 7.4 (phosphate buffer, ionic strength 0.3) at six various temperatures between 2 and 88° C.

All spectra have been taken on a Varian HA-100 spectrometer equipped with a variable temperature system. The temperatures have been calculated from the methanol- and glycol-shifts (Van Geet, 1963 and 1970).

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

In this paper, the determination of the thermodynamic quantities of self-association is based on the isodesmic model: each attachment of a monomer A_1

(concentration a_1) to an associate A_n (concentration a_n) of n molecules A_1 has the same equilibrium constant K.

$$A_n + A_1 \rightleftharpoons A_{n+1}$$

$$K = \frac{a_{n+1}}{a_n\, a_1} = \text{const.} \qquad n = 1, 2, 3, \ldots$$

With two additional assumptions: $n \to \infty$ and $x = K \cdot a_1 < 1$ K and x are related according to

$$K = \frac{1}{a_0} \cdot \frac{x}{(1-x)^2} \tag{1}$$

$$x = 1 + \frac{1}{2\,x_0}\left(1 - \sqrt{1 + 4\,x_0}\right) \tag{2}$$

whereas $x_0 = K \cdot a_0$ (a_0: total concentration).

A difficulty occurring in thermodynamic NMR studies is the calculation of x using the observed chemical shift δ observed for a proton which, normally, is the average of the individual chemical shifts of that proton in all associates A_n. This problem may be simplified by considering only three different chemical shifts:

$$\delta = g_1\,\delta_1 + g_2\,\delta_2 + g_3\,\delta_3$$

or, since $\sum g_i = 1$:

$$\Delta = g_2\,\Delta_2 + g_3\,\Delta_3$$

with $\Delta = \delta - \delta_1$, $\Delta_i = \delta_i - \delta_1$, and $\delta_1 \equiv \delta_M$ ("monomer shift"). Δ_2 is the relative chemical shift in the dimer complex ("dimer shift"). The protons in both of the molecules at the ends of an associate A_n have the same complex shift Δ_2; therefore,

$$g_2 = \sum_{n=2} 2\,\frac{a_n}{a_0}\,.$$

The protons in the other $(n-2)$ molecules in the associate have the average complex shift Δ_3 ("trimer shift") resulting from the additive superposition of the magnetic anisotropy of the neighbouring molecules:

$$g_3 = \sum_{n=2} (n-2)\,\frac{a_n}{a_0}\,.$$

Antonovsky *et al.* (1973) suppose $\Delta_3 = \Delta_2$ that is, they assume an average chemical shift Δ_s of the protons in all of the associates while Dimicoli and Helene (1973) consider the special case of $\Delta_3 = 2\,\Delta_2$.

Using $\alpha = \Delta/\Delta_2$ and $\gamma = \Delta_3/\Delta_2$ the following equations can be derived:

$$\alpha = (2-\gamma) + [2 - (2-\gamma)\,(2 + 1/x_0)] \cdot x \tag{3}$$

$$x = \frac{\alpha}{1 + \sqrt{1 - (2-\gamma)\,\alpha}}\,. \tag{4}$$

According to Eqs. (1) and (4) K can be calculated from the chemical shift δ observed for a proton if the following three NMR parameters of that proton are known: the monomer shift δ_M, the dimer shift Δ_2, and the quotient γ (oı the trimer shift). Whereas δ_M can be determined, to a certain degree, by graphical extrapolation, a prediction for the absolute size of Δ_2 and Δ_3 as well cannot be given. The quotient γ, however, should not be too large since a value near two seems to be reasonable according to the considerations of Dimicoli and Helene (1973). Obviously, the dimer shift Δ_2 is, like K, a free parameter and was used, therefore, in this study for minimizing the average deviation F of the shifts calculated from the observed values at each temperature measured according to Eqs. (2) and (3):

$$F\,(\delta_M, \Delta_2, \gamma, K) := \frac{1}{N}\left[\sum_{n=1}^{N} (\Delta^{(n)}_{\text{observed}} - \Delta^{(n)}_{\text{calculated}})^2\right]^{1/2}.$$

N: number of samples.

The minimum of F determines the K values. The temperature dependence of these has been used for calculating the enthalpies and entropies according to the van't Hoff equation.

An exact determination of chemical shifts requires an appropriate reference substance. Using an internal reference the frequencies of reference and solute as well are altered due to interactions between them. When the frequencies are measured relative to an external standard changes of the susceptibility of the solution caused by the solute must be taken into account. In order to avoid interactions between reference and solute the chemical shifts observed in this investigation have been measured relative to the H_2O lock and, then, have been reduced to shifts relative to TPA measured in a separate probe.

Results and Discussion

The chemical shifts of the various protons of a compound are differently influenced if the concentration of the compound and/or the temperature of the sample are varied. The evaluation according to the model used results then, generally, in temperature dependent values of Δ_2 and K which are different for the various protons observed.

The temperature dependence of Δ_2 can be approximated, in general, by linear functions (max. 0.3 Hz deg^{-1}). Furthermore, the absolute size of Δ_2 depends on the values of γ and δ_M as well. At $\gamma = 2$ and 0° C the Δ_2 values vary from 10 Hz (CH_3-10 of $m_2^6e^9$Ade) to 60 Hz (H-2 of m^6m^9Ade). The relative size of Δ_2 of the various protons of one molecule seems to reflect the different sensitivities of the protons (Lüdemann and von Goldammer, 1973).

The temperature dependence of K satisfies the van't Hoff equation (correlation factors > 0.99). Therefore, it is possible to calculate the enthalpy ΔH and the entropy ΔS from the chemical shifts of each proton. K, ΔH, and ΔS depend on the values of γ and δ_M as well.

When the quotient γ is varied, at a constant δ_M, ΔH as well as K depend on γ as shown in Fig. 1 for the H-2 proton of m^6m^9Ade. Qualitatively, the same curves have been obtained for all of the protons of m^6m^9Ade and $m_2^6e^9$Ade. K increases almost linearly with γ, whereas ΔH and (not shown) ΔS increase up to $\gamma \approx 2$ and are about constant for $2 \leq \gamma \leq 4$. In this region of γ also the average deviation F is about constant and is considerably less than for $\gamma < 2$. Therefore, the enthalpy and the entropy are calculated at $\gamma = 2$. It should be emphasized, however, that the K values depend strongly on γ.

If, on the other hand, δ_M is varied at a constant value of γ, K increases and ΔH decreases with increasing δ_M (Fig. 2). The deviation F has a pronounced minimum. Similar curves have been obtained for all of the other protons of the compounds if the parameter δ_M was chosen independently of temperature and, then, was varied.

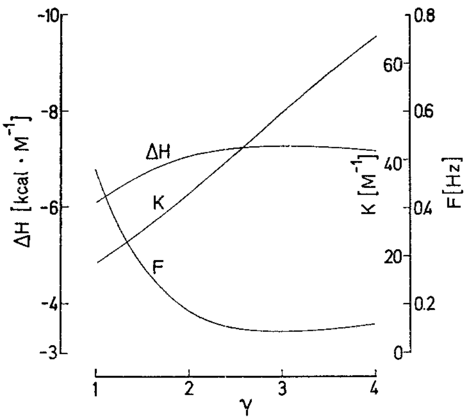


Fig. 1

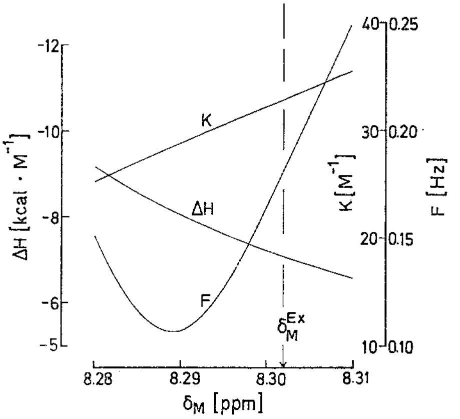


Fig. 2

Fig. 1. Enthalpy ΔH and apparent equilibrium constant K at 25° C of the H-2 proton of m^6m^9 Ade versus γ at $\delta_M = 8.302$ ppm. F: average deviation of the fit

Fig. 2. Enthalpy ΔH and apparent equilibrium constant K at 25° C of the H-2 proton of m^6m^9 Ade versus δ_M at $\gamma = 2$. F: average deviation of the fit. δ_M^{Ex}: monomer shift extrapolated at 88° C

The monomer shift δ_M is usually obtained by graphical extrapolation to zero concentration. This is, however, very difficult in the case of a strongly curved function $\delta(a_0)$ in the lower concentration range (that is large K values). Therefore, δ_M has been varied in order to obtain an optimum fit according to Eq. (3) using a computer. For some protons, these δ_M values shifted 0.2 Hz · deg^{-1} or more downfield with increasing temperature. Since this temperature dependence was related to the concentration range selected for the extrapolation the assumption seems to be reasonable that the effect can be simulated by the evaluation method. This has been confirmed by measurements concerning the temperature dependence of δ_M.

Like in the case of the other NMR parameters also the monomer shift δ_M seems to be temperature dependent. Since water interacts, at least, with the solute by hydrogen bonds the δ_M values are the chemical shifts of monomers complexed with surrounding water molecules. These complex shifts will be dependent on temperature, especially, when the observed proton is directly involved in a hydrogen bond.

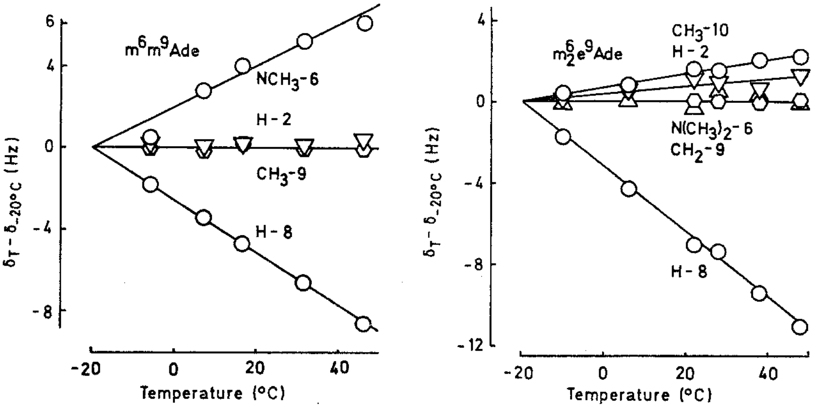
In order to determine the influence of bound water, m^6m^9Ade and $m_2^6e^9$Ade have been measured in methanol. A concentration dependence of the chemical shifts (i.e. stacking) could not be detected. Since the solvent shifts of m^6m^9Ade and $m_2^6e^9$Ade relative to CCl_4 in phosphate buffer (pD 7.4) and in methanol are of the same direction and order of magnitude (Table 1) it can be concluded that the solvent-solute interactions in water and methanol are similar. Therefore, the temperature dependence of the chemical shifts in methanol should resemble that one of the monomer shifts in water.

As can be seen from Fig. 3 only the chemical shift of H-8 of m^6m^9Ade and $m_2^6e^9$Ade is moved upfield 0.1 to 0.2 Hz · deg^{-1} while the one of NCH_3-6 of m^6m^9Ade

Table 1. The monomer shifts of m^6m^9 Ade and $m_2{}^6e^9$ Ade, resp., in methanol and phosphate buffer (pD 7.4) relative to the chemical shifts in CCl_4

m^6m^9Ade	H-2	H-8	NCH_3-6	CH_3-9	
CCl_4	8.185	7.486	3.201	3.787	ppm
Methanol	+6.3	+51.3	-8.4	+1.6	Hz
D_2O	+11.7	+55.1	-7.0	+2.7	Hz

$m_2^6e^9$Ade	H-2	H-8	$N(CH_3)_2$-6	CH_2-9	CH_3-10	
CCl_4	8.099	7.512	3.495	4.191	1.509	ppm
Methanol	+10.3	+50.0	-0.4	+5.7	-4.7	Hz
D_2O	+13.3	+56.6	-4.1	+4.9	-4.1	Hz



a b

Fig. 3. Temperature dependence of the chemical shifts of m^6m^9 Ade (3a) and $m_2{}^6e^9$ Ade (3b) in methanol relative to chemical shifts at − 20° C

is moved downfield 0.1 Hz · deg^{-1} with increasing temperature. The chemical shifts of the other protons are more or less independent of temperature. The response of the H-8 shift confirms the suggestion of Hruska *et al.* that this proton is directly hydrogen bonded to solvent molecules (Hruska *et al.*, 1968). The downfield shift of the NCH_3-6 protons results from hydrogen bonds of the neighbouring NH-6 to the solvent.

In any case, it is evident that downfield shifts of about 0.2 Hz · deg^{-1} or more with increasing temperature are caused by the computer extrapolation. Therefore, the thermodynamic quantities have been calculated, in general, with δ_M values independent of temperature and extrapolated at the highest temperature used as has been done e.g. by Antonovsky *et al.* (1973). Only those protons whose δ_M has been observed to be temperature dependent in methanol have been evaluated with δ_M dependent and independent of temperature.

The thermodynamic quantities of m^6m^9Ade and $m_2^6e^9$Ade calculated with $\gamma = 2$ are summarized in Table 2. The apparent equilibrium constants K calculated from

Table 2. Apparent equilibrium constant K at 25° C and enthalpy ΔH of selfassociation of m^6m^9 Ade and $m_2{}^6e^9$ Ade in phosphate buffer (pD 7.4) at $\gamma = 2.f(T)$: the monomer shift δ_M taken as temperature dependent

m^6m^9Ade	H-2	H-8		NCH_3-6		CH_3-9
δ_M [ppm]	8.302	8.037	f(T)	3.131	f(T)	3.814
$K^{25°C}$[M^{-1}]	32.9	22.1	50.9	23.0	14.8	18.5
ΔH[kcal·mol^{-1}]	-7.1	-7.0	-6.1	-7.7	-6.5	-7.1

$m_2^6e^9$Ade	H-2	H-8		$N(CH_3)_2$-6	CH_2-9	CH_3-10
δ_M [ppm]	8.232	8.078	f(T)	3.454	4.240	1.468
$K^{25°C}$[M^{-1}]	73.4	42.1	107.9	47.3	41.6	29.2
ΔH[kcal·mol^{-1}]	-7.5	-7.4	-7.1	-6.8	-7.3	-7.2

the chemical shifts of different protons of the same molecule disagree by a factor of two or more. The corresponding enthalpies, however, are about -7 kcal · mol^{-1} for all protons of both of the substances. The enthalpy of a proton is slightly less if δ_M depends on the temperature. The entropies of the different protons of m^6m^9Ade and $m_2^6e^9$Ade are about -18 e.u. and -17 e.u., resp.

Whereas the thermodynamic quantities of $m_2^6e^9$Ade, to our knowledge, have not yet been measured those of m^6m^9Ade have been determined by several authors using different experimental techniques. Pörschke and Eggers (1973) have obtained the values $\Delta H = -8.7 \pm 1.5$ kcal · mol^{-1} and $\Delta S = -21.6 \pm 3$ e.u. for m^6m^9Ade using vapor pressure osmometry; similar values were obtained by Bretz *et al.* (1974) using equilibrium ultracentrifugation. Antonovsky *et al.* (1973) have obtained $\Delta H = -8.2 \pm 0.6$ kcal · mol^{-1} and $\Delta S = -21.0$ e.u. by means of NMR technique but at $\gamma = 1$.

The errors of ΔH and ΔS calculated from the chemical shift of one proton are caused mainly by the error of the δ_M value. Considering also all of the other experimental errors depending essentially on the range of the saturation factor s (as defined in analogy to Deranleau (1969) $s :=$ complex concentration to weighted concentration) the average error of ΔH of one proton is about $\pm$ 15% and that of ΔS is about $\pm$ 20%. The errors of ΔH and ΔS averaged from the values of the various protons are about $\pm$ 8% and $\pm$ 10% resp. Therefore, the enthalpy of selfassociation of m^6m^9Ade as well as of $m_2^6e^9$Ade as determined in the present investigation is $\Delta H = -7.2 \pm 0.6$ kcal mol^{-1}, the entropies are $\Delta S = -17.9 \pm 1.8$ e.u. for m^6m^9Ade and $\Delta S = -16.7 \pm 1.7$ e.u. for $m_2^6e^9$ Ade.

Conclusions

Using NMR data for evaluating the selfassociation of nucleobases in aqueous solutions each proton of a substance has to be considered separately. The temperature dependence of the monomer shift δ_M may be simulated by the evaluation method and has to be confirmed by separate experiments. The values of Δ_2 and K are different for the various protons of a molecule. The temperature dependence of these K values, however, satisfies the van't Hoff equation and yields an enthalpy

ΔH and an entropy ΔS which remain about constant for all protons observed within the experimental errors.

The average deviation F of the fit is minimal for $2 \leq \gamma \leq 4$. Whereas K depends strongly on γ, ΔH and ΔS are about constant in this region of γ. Therefore, the thermodynamic quantities ΔH and ΔS can be calculated according to the model of Dimicoli and Helene (1973) using $\gamma = 2$.

Acknowledgement. This work was supported in part by the Fraunhofer Gesellschaft.

References

Antonovsky, V. L., Gukovskaja, A. S., Nekrasova, G. V., Sukhorukov, B. J., Tchervin, I. I.: Studies of intermolecular interactions of nucleic acid bases in aqueous solutions by the proton magnetic resonance method. Selfassociation of 6,9-dimethyladenine and its pyrazole analogue and their association with methyl-substituted cytosine and uracil. Biochim. biophys. Acta (Amst.) **331**, 9—20 (1973)

Bretz, R., Lustig, A., Schwarz, G.: Selfassociation studies of two adenine derivatives by equilibrium ultracentrifugation. Biophys. Chem. **1**, 237—241 (1974)

Deranleau, D. A.: Theory of the measurement of weak molecular complexes. I. General considerations. J. Amer. chem. Soc. **91**, 4044—49 (1969)

Dimicoli, J. L., Helene, C.: Complex formation between purine and indole derivatives in aqueous solutions. Proton magnetic resonance studies. J. Amer. chem. Soc. **95**, 1036—44 (1973)

Hruska, F. E., Bell, C. L., Victor, T. A., Danyluk, S. S.: Medium effects on the nuclear magnetic resonance spectra of purines. Biochemistry **7**, 3721—27 (1968)

Lawaczeck, R.: Stacking Assoziate der decarboxylierten aromatischen Aminosäuren mit Mono- und Oligonucleotiden. Ph.D. thesis, Technische Universität Braunschweig 1972

Lüdemann, H. D., von Goldammer, E.: The association between purine nucleosides and benzene in aqueous solution, studied by proton magnetic resonance and nuclear Overhauser enhancements. Z. Naturforsch. **28c**, 361—369 (1973)

Marenchic, M. G., Sturtevant, J. M.: Calorimetric investigation of the association of various purine bases in aqueous media. J. physic. Chem. **77**, 544—548 (1973)

Pörschke, D., Eggers, F.: Thermodynamics and kinetics of base stacking interactions. Europ. J. Biochem. **26**, 490—498 (1972)

Ts'o, P. O. P.: Fine structure of proteins and nucleic acids (eds. G. D. Fasman, S. N. Timasheff). New York: Marcel Dekker 1970

Van Geet, A. L.: Calibration of the methanol and glycol nuclear magnetic resonance thermometers with a static thermistor probe. Anal. Chem. **40**, 2227—29 (1963)

Van Geet, A. L.: Calibration of methanol nuclear magnetic resonance thermometer at low temperature. Anal. Chem. **42**, 679—680 (1970)

Professor Dr. Wolfgang Lohmann
Institut für Biophysik der Universität
Strahlenzentrum, Leihgesterner Weg 217
D-63 Giessen
Federal Republic of Germany