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MASS-SPECTROMETRIC INVESTIGATIONS ON HYDRATION OF NUCLEIC ACID COMPONENTS IN VACUUM

II. *N*-METHYLATED ADENINES

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Association reactions between water and *N*-methylated adenines (M), occurring under field-ionization conditions in a mass spectrometer at the tungsten point emitter surface, were studied at a number of temperatures. The origin of the peaks observed in the mass spectra at *m/e* ratios corresponding to MH^+ was assigned to $M \cdot H_2O$ monohydrates, of those corresponding to $(MH \cdot H_2O)^+$ and $(MH_2)^{2+}$ to double hydrates $M(H_2O)_3$. Measurements of the relative peak intensities allowed calculation of apparent equilibrium constants, K_{ass}, for successive hydration steps, and hence the respective van't Hoff enthalpies of hydration. Both the equilibrium and the energy parameters obtained support the available quantum-mechanical single-layer hydration schemes of adenine and of its *N*-methylated derivatives.

1. Introduction

It is now well established that hydration of nucleic acids plays a crucial role in determination of their conformational properties and in functional interactions involving proteins and other ligand molecules. Until recently, however, our knowledge concerning the hydration properties of particular nucleic acid bases was largely limited to quantum-chemical studies [1–6]. In order to gain also necessary experimental information on this point we recently studied enthalpies of hydration of alkylated uracils in water [7,8] and in a vacuum [9] using the field-ionization mass spectrometric method [10]. This method proved to be fully applicable in studies of vapour phase association equilibria involving nucleic acid bases [10] and water [11]. In the present paper we report results of similar studies on association reactions between *N*-methylated adenines and water, occurring at the field emitter surface of a mass spectrometer.

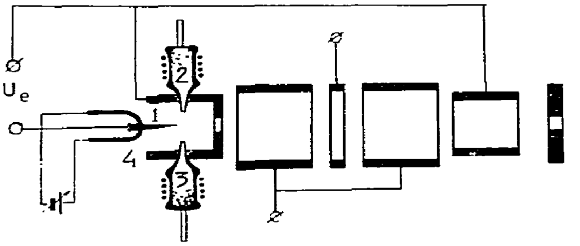

Fig. 1. Scheme of field-ionization ion source (for explanation see text).

2. Materials and methods

All experiments were carried out on a mass spectrometer model MI-1201 (U.S.S.R.), equipped with a field-ionization source designed for our particular purposes as shown schematically in fig.

1. The main element of the source if a metal point emitter 1, with a radius of curvature $r_e = 4000$ Å, formed by electrochemical etching in NaOH of the tungsten wire of 0.15 mm diameter. Under our operational conditions the emitter potential was set at $U_e = 5$ kV so that the electric field strength near the point tip was $F_e = U_e/5r_e = 0.25$ V/Å. At this field strength the ionization of particles occurs most probably by tunneling of an electron from a molecule into the metal electrode. Water and the compounds under examination were supplied to the emitter from two glass evaporators 2 and 3, heated by nichrome coils. The temperature of the emitter was thus controlled by the temperature of evaporator 3: when the latter was set at 353–363 K, the former approached 303–313 K. Use of the crystalline hydrate $CuSO_4 \cdot 5H_2O$ as the water source allowed maintenance of a stable flow of water vapour in vacuum ($P = 10^{-8}$ torr) over a long period of time. The counterelectrode, 4, had a negative potential $U_{re} = -(0–7)$ kV.

The principle of the method and the chemical equilibrium conditions at the surface of the point emitter are discussed elsewhere [10].

The investigated *N*-methylated adenines (7-methyladenine (m^{7}Ade), 9-methyladenine (m^{9}Ade), 6,6-dimethyladenine (m$^{6,6}_2$Ade), 6,9-dimethyladenine (m$^{6,9}_2$Ade) and 6,6,9-trimethyladenine (m$^{6,6,9}_3$Ade)) were synthesized according to standard methods and purified by repeated crystallization from water and vacuum sublimation.

3. Results

The field-ionization mass spectra of the methylated adenines were obtained at several intensities (i_e) of the electric current flow through the emitter, in order to determine variation of the water-base equilibria with temperature and the respective vant'Hoff enthalpies of hydration therefrom. In the first series of experiments, involving simultaneously m^{9}Ade and m$^{6,9}_2$Ade, transformation of the measured ion current J dependence on the electric current intensity, $\log J = f(i_e)$, into the desired function, $\log J = f(T_e)$, of the emitter temperature T_e was achieved by placing in the evaporator dimethyluracil (m$^{1,3}_2$Ura), for which hydration equi-

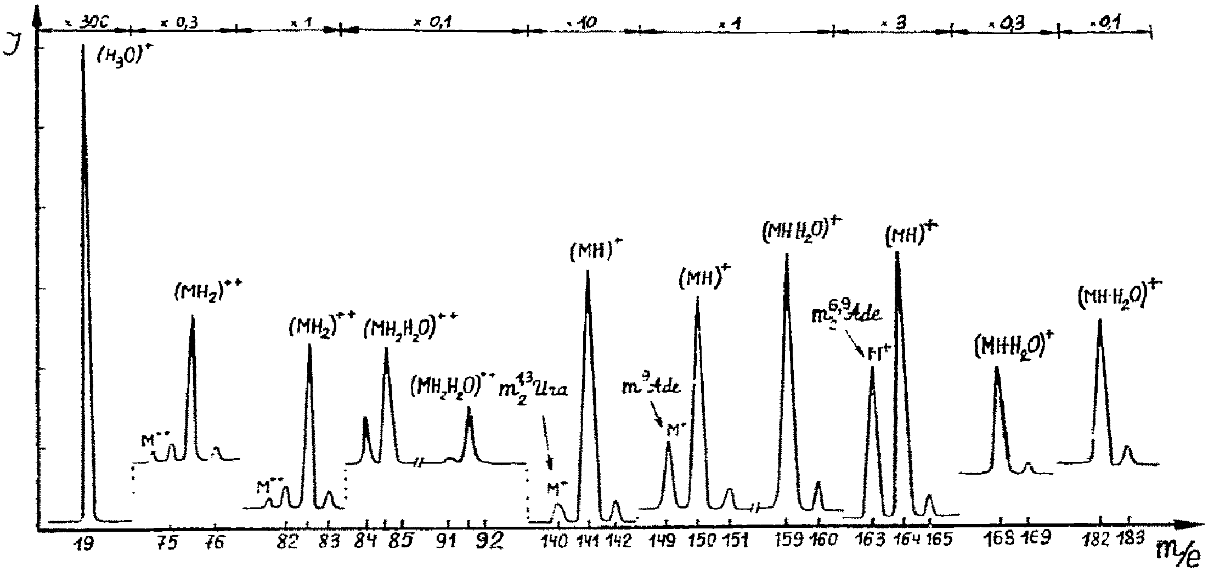

Fig. 2. Field-ionization mass spectrum of the ternary mixture: m$^{1,3}_2$Ura, m^{9}Ade and m$^{6,9}_2$Ade, in the presence of water vapour at 293 K.

librium at a number of temperatures had been thoroughly studied previously [9]. This compound was thus used as in internal 'temperature indicator'. In the subsequent experimental series pairs of methylated adenines were studied likewise, one of which, included also in the preceding series, served now as the 'secondary temperature indicator'.

Recording of the mass spectra for pairs of compounds simultaneously in one experiment, i.e., with the same source geometry and emitter temperature, permitted also direct comparison of apparent association constants for hydration of the bases, derived as discussed in section 3.2.

3.1. Field-ionization mass spectra at 293 K

The mass spectrum of the ternary system, $m^9Ade + m_2^{6,9}Ade + m_2^{1,3}Ura$ (fig. 2), is characterized by the presence of two intense peaks of protonated ions MH^+ (at *m/e* 150 and 164 of m^9Ade and $m_2^{6,9}Ade$, respectively), resulting from ionization of neutral $M \cdot H_2O$ hydrates accompanied by subsequent proton transfer from the water molecule onto a base (cf. fig. 3 in the preceding paper [9]). The spectrum exhibits also peaks at *m/e* 168 and 182 of $(MH \cdot H_2O)^+$ originating from field-ionization of $M(H_2O)_2$ double hydrates. These ions arise according to the same field-ionization mechanism postulated for formation of $(MH)^+$.

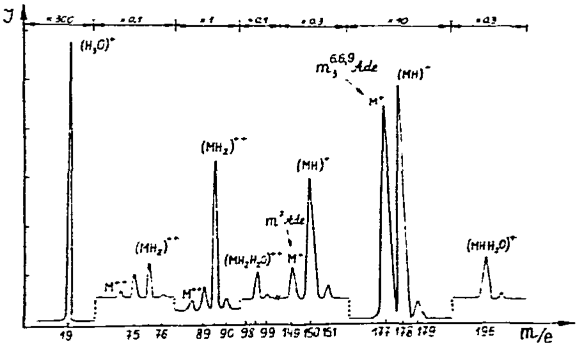

Fig. 4. Field-ionization mass spectrum of binary mixture: m^7Ade and $m_3^{6,6,9}Ade$, in the presence of water vapour at 293 K.

Analysis of variation of the ion currents $J_{(MH)^+}$ and $J_{(MH \cdot H_2O)^+}$ with concentration of water ($J_{H_2O^+}$, $J_{H_3O^+}$) in the reaction zone demonstrated that both mono- and dihydrates of the bases were formed in the first-order reaction with respect to water. This means that binding of water by the bases is a multi-step single water molecule addition reaction:

$$M + H_2O \rightleftharpoons M \cdot H_2O \xrightarrow{H_2O} M(H_2O)_2 \xrightarrow{H_2O} M(H_2O)_3.$$

The mass spectrum of the binary mixture, $m^9Ade + m_2^{6,9}Ade$ (fig. 2), as well as the spectra of other binary mixtures of the bases (figs. 3–5)

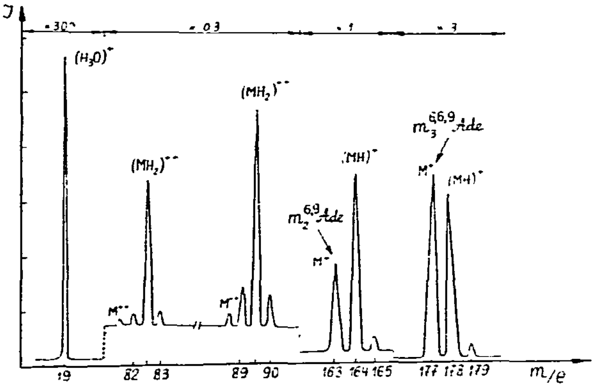

Fig. 3. Field-ionization mass spectrum of binary mixture: $m_2^{6,9}Ade$ and $m_3^{6,6,9}Ade$, in the presence of water vapour at 293 K.

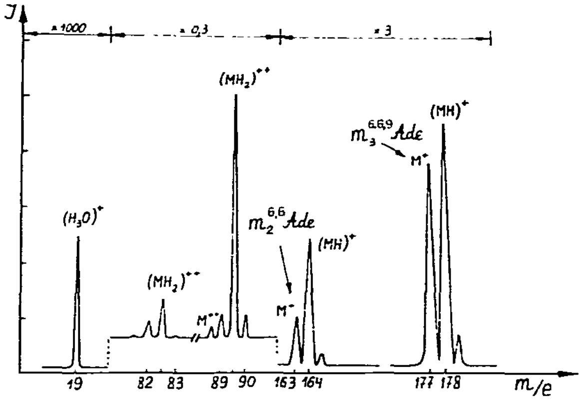

Fig. 5. Field-ionization mass spectrum of binary mixture: $m_2^{6,6}Ade$ and $m_3^{6,6,9}Ade$, in the presence of water vapour at 293 K.

taken in the presence of water indicate occurrence of a series of doubly charged ions: M^{2+}, $(MH)^{2+}$, $(MH_2)^{2+}$ and $(MH \cdot H_2O)^{2+}$. These ions were not observed in the mass spectra of alkylated uracils obtained under similar electric-field conditions in the presence of water vapour [9]. The appearance of doubly charged ions of methylated adenines upon field ionization is due undoubtedly to the relatively low ionization potential of adenine as compared to that of diketopyrimidines [12–14]. This difference in ionization potential, concerning the highest occupied electron orbital, between these two groups of bases is also well reflected in their protonation equilibria in aqueous solution [15].

In all probability the doubly charged ions are formed by successive ionization of hydrated clusters $M(H_2O)_n$ ($n = 1, 2, 3$) as shown schematically below:

$$M \cdot H_2O + H_2O \leftrightharpoons M(H_2O)_2 \xrightarrow{\text{ionization } e^-} (MH \cdot H_2O)^+ + OH \xrightarrow{\text{ionization } e^-} (MH_2)^{2+} + OH \qquad (1)$$

$(MH_2 \cdot H_2O)^{2+}$ is generated according to an analogous scheme from triple $(M(H_2O)_3$ hydrates.

The mass spectra of m$^{6,9}_2$Ade + m$^{6,6,9}_3$Ade (fig. 3), m^{7}Ade + m$^{6,6,9}_3$Ade (fig. 4) and of m$^{6,6}_2$Ade + m$^{6,6,9}_3$Ade (fig. 5) are self-explanatory in connection with the preceding discussion and deserve only one comment. In the mass spectrum of m$^{6,6,9}_3$Ade (fig. 3) the peaks corresponding to the protonated base $(MH)^+$ and to its molecular ion M^+ are comparable in intensity, while in the spectra of all other compounds the peak intensity of the $(MH)^+$ is always distinctly higher than that of the molecular ion M^+. This is due to less favourable equilibrium and energy parameters of hydration for m$^{6,6,9}_3$Ade as compared to those for partially methylated adenine derivatives, as will be shown below.

3.2. *Evaluation of equilibrium constants for base-water association*

The apparent association constants $K_{\text{ass}}^{M \cdot H_2O}$ (shown in table 2) for formation of monohydrates of the bases were calculated as the ratios of peak intensities of appropriate ionic species in the mass spectra (figs. 2–5), proportional to the concentration in the reaction zone of neutral molecules involved in the hydration equilibrium:

$$K_{\text{ass}}^{M \cdot H_2O} = \frac{[M \cdot H_2O]}{[M] \cdot [H_2O]} = \frac{J_{(MH)^+}}{J_{M^+} \cdot J_{H_3O^+}} \qquad (2)$$

In these calculations, instead of the weak $J_{H_2O^+}$ ion current, a much more intense ion current $J_{H_3O^+}$ was used which was proportional to $J_{H_2O^+}$ [11].

$K_{\text{ass}}^{M(H_2O)_2}$ values characterizing addition of the second water molecule to $M \cdot H_2O$ complexes were calculated analogously from peak intensities of $(MH \cdot H_2O)^+$, $(MH_2)^{2+}$, $(MH)^+$ and H_3O^+ in the mass spectra (figs. 3–5). They are listed also in table 1.

Table 1

Apparent association constants of formation of mono-, di- and trihydrates of *N*-methylated adenines at 293 K, derived from mass-spectroscopic line intensities of ionic species in figs. 2–5 (in parentheses)

Base	K_{ass} ($\times 10^5$) (arbitrary units)		
Hydrate:	$M \cdot H_2O$	$M(H_2O)_2$	$M(H_2O)_3$
m$^{1,3}_2$Ura	30.0 (2)	–	–
m^{7}Ade	8.6 (4)	0.22 (4)	–
m^{9}Ade	7.1 (2)	0.81 (2)	0.32 (2)
m$^{6,6}_2$Ade	4.7 (5)	0.056 (5)	–
m$^{6,9}_2$Ade	$\overline{4.35}$ 4.8 (2) 3.9 (3)	$\overline{0.54}$ 0.58 (2) 0.50 (3)	0.075 (4)
m$^{6,6,9}_3$Ade	$\overline{2.2}$ 2.1 (3) 2.5 (4) 2.0 (5)	$\overline{0.21}$ 0.31 (5) 0.16 (4) 0.17 (3)	0.039 (2)

Taking into account peak intensities of all the ionization products of the neutral species involved in the process of addition of the third water molecule to M(H_2O)$_2$ complexes of m^{9}Ade, m$_2^{6,9}$Ade and m$_3^{6,6,9}$Ade (figs. 2 and 4), respective apparent association constants $K_{\text{ass}}^{\text{M(H}_2\text{O)}_3}$ were also evaluated (table 1).

The apparent association constants thus obtained are proportional to the true association constants [10]:

$$K_{\text{ass,app}}^{\text{M(H}_2\text{O)}_n} = \delta_{\text{eff}}(F) \cdot K_{\text{ass,true}}^{\text{M(H}_2\text{O)}_n} \tag{3}$$

The proportionality factor $\delta_{\text{eff}}(F)$ in eq. 3 is the effective ionization coefficient at a given field strength F, defined as an appropriate ratio of the ionization coefficients $\delta_i(F)$ of particular neutral molecules involved in the hydration equilibrium. The ionic currents $J_i(F)$ are proportional to molecular concentrations $n_i(F)$ in the ionization zone [10], i.e., $J_i(F) = \delta_i(F) \cdot n_i(F)$. Hence, the effective ionization coefficient in eq. 3 written, for example, for $K_{\text{ass}}^{\text{M(H}_2\text{O)}}$ is given as:

$$\delta_{\text{eff}}(F) = \frac{\delta \text{M} \cdot \text{H}_2\text{O}(F)}{\delta_{\text{M}}(F) \cdot \delta_{\text{H}_2\text{O}}(F)}.$$

The ionization coefficients at a given field strength and emitter temperature may vary from experiment to experiment largely owing to changes in the source geometry [10]. Therefore, the apparent association constants for a series of compounds can be compared, provided that they are obtained under the same experimental conditions and that the ionization potentials of the compounds are closely comparable. The latter condition seems to be quite satisfied in the case of the homologous series of N-methylated adenines since, judging from the data for alkylated uracils [12,14], the ionization potential of adenine is not expected

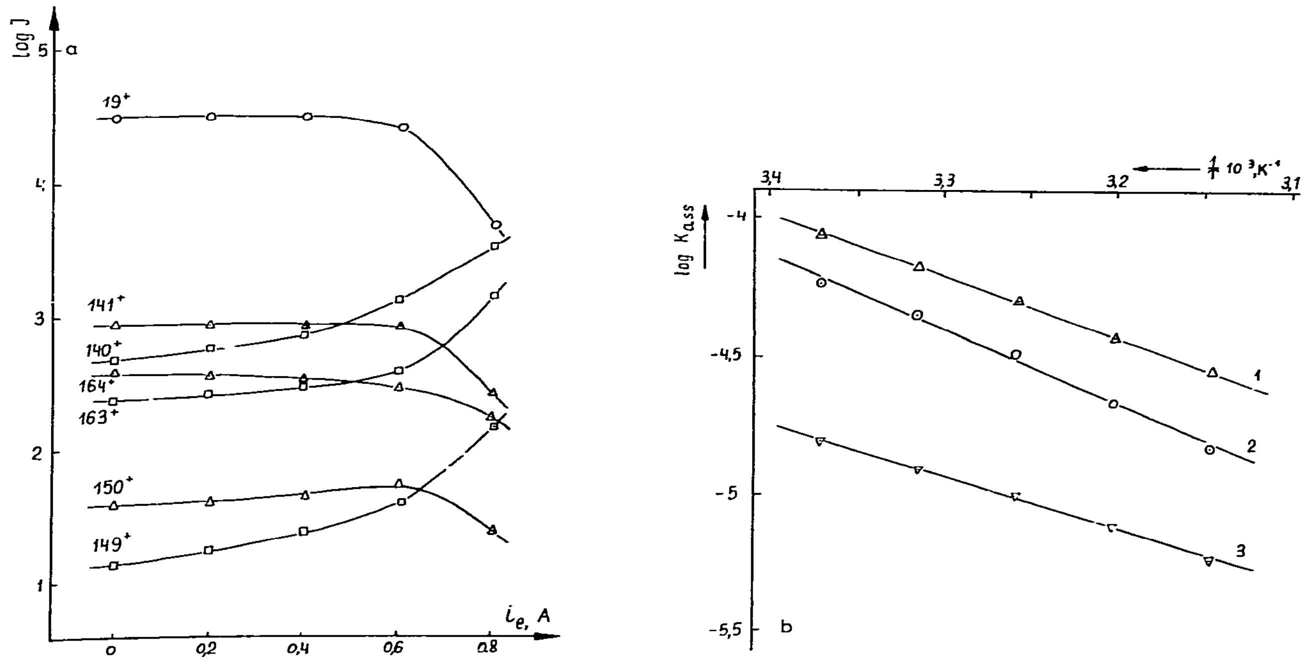

Fig. 6. (a) Temperature dependence of intensity J of ionic currents corresponding to ionic species in the mass spectrum of the ternary mixture: m$_2^{1,3}$Ura, m^{9}Ade and m$_2^{6,9}$Ade, taken in the presence of water vapour (cf. fig. 2); at $i_e = 0$ A the temperature of the emitter surface $T_e = 313$ K. (b) Van't Hoff plots of K_{ass} for M·H_2O complexes of m^{9}Ade(1), m$_2^{1,3}$Ura(2) and m$_2^{6,9}$Ade(3); the latter curve is shifted along the ordinate by $\Delta \log J = -0.5$.

to vary significantly with the site of single *N*-methyl substitution. The fulfillment of the first condition was attempted by simultaneous field ionization in one mass-spectrometric experiment of pairs of the bases. They were arranged from the series of the compounds investigated in such a way that first the mass spectrum for m$^{6,9}_{2}$Ade and m$^{6,6,9}_{3}$Ade was obtained (fig. 3), and then in subsequent experiments one of these bases was always included as an 'internal standard'. The association constants $K_{\mathrm{ass}}^{\mathrm{M(H_2O)}}$ and $K_{\mathrm{ass}}^{\mathrm{M(H_2O)_2}}$ obtained for the two bases in separate experiments in most cases do not deviate from the average values by more than about 10–20% (cf. table 1). So that the relative order of apparent association constants for formation of mono- and dihydrates of the bases could be reasonably established for the whole series of *N*-methylated adenines. However, such a comparison of apparent association constants for stepwise hydration of a given compound is not justified because of the very different $\delta_i(F)$ expected for singly and doubly ionized species, the ionic currents of which were used in calculations of equilibrium parameters.

3.3. Evaluation of van't Hoff enthalpies of association

In fig. 6a log J versus i_e plots, equivalent to temperature dependence of the mass spectrum line intensities in the temperature range 298–318 K, are shown for singly charged M^+ and $(MH)^+$ of m$^{1,3}_{2}$Ura (*m/e* 140 and 141), m^{9}Ade (*m/e* 149 and 150), m$^{6,9}_{2}$Ade (*m/e* 163 and 164) and of H_3O^+ (*m/e* 19): within such a relatively narrow temperature range ionization coefficients can be regarded as practically constant [10]. Therefore, ionic currents are determined by temperature-dependent

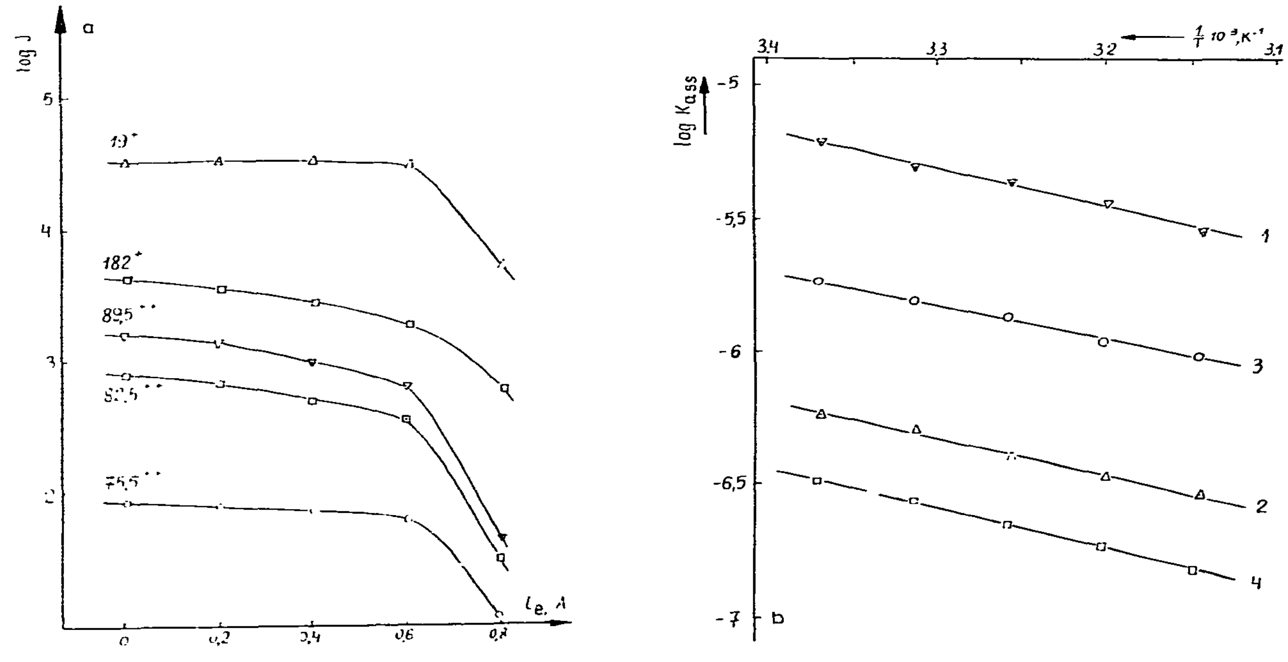

Fig. 7. (a) Temperature dependence of intensity J of ionic currents corresponding to $(MH_2)^{2+}$ and $(MH\cdot H_2O)^+$ species in the mass spectra of m^{9}Ade (75.5^{++}), m$^{6,6,9}_{3}$Ade (89.5^{++}) and m$^{6}_{2}$Ade (82.5^{++} and 182^{+}) taken in the presence of water vapour (cf. figs. 2 and 3); the curve for *m/e* 182$^+$ is shifted along the ordinate by Δ log J = 3; temperature of the emitter surface T_e = 313 K at i_e = 0 A. (b) Van't Hoff plots of K_{ass} for $M(H_2O)_2$ complexes of m^{9}Ade (1), m$^{6,9}_{2}$Ade (curves 2 and 4 corresponding to $K_{\mathrm{ass}}^{\mathrm{M(H_2O)_2}}$ obtained with the use of $J_{82.5^{++}}$ and $J_{182^{+}}$, respectively) and m$^{6,6,9}_{3}$Ade (3); curve 2 is shifted along the ordinate by Δ log J = −1.

Table 2

Enthalpies of formation in vacuum, ΔH_{hydr}, of mono-, di- and trihydrates of the bases (M) and corresponding energies of water binding, E, calculated theoretically [20]

Values are expressed in kJ mol^{-1}.

Base	Hydrate				
	$M \cdot H_2O$		$M(H_2O)_2$		$M(H_2O)_3$
	$-\Delta H_{hydr}$	$-E$	$-\Delta H_{hydr}$	$-E$	$-\Delta H_{hydr}$
m^7Ade	46.4 (±6.3)	53.6	–	36.8	–
m^9Ade	44.3 (±4.2)	41.4	32.4 (±5.0)	35.6	30.1 (±5.4)
$m_2^{6,6}$Ade	42.2 (±3.3	37.7	–	46.0	–
$m^{6,9}$Ade	38.5 (±5.0)	41.1	29.5 (±5.4)	33.5	25.1 [a]
$m_3^{6,6,9}$Ade	34.7 (±3.3)	32.6	28.0 (±4.6)	32.6	24.7 [a]

[a] Values estimated from the ratio of K_{ass} for formation of di- and trihydrates upon assumption of similar entropy changes in both processes.

concentrations of neutral molecules involved in the hydration equilibria. As evidenced by the quasi-plateau attained by log $J = f(i_e)$ curves for all the ions, each point thereon corresponds to an equilibrium state of the hydration reaction in the reaction zone. The apparent association constants $K_{ass}^{M \cdot H_2O}$ obtained at various temperatures from these data are plotted according to the van't Hoff equation in fig. 6b. Linear van't Hoff plots were likewise obtained for $K_{ass}^{M \cdot H_2O}$ of other bases: m^7Ade, $m_2^{6,6}$Ade and $m_3^{6,6,9}$Ade (data not shown). The enthalpies of hydration ΔH_{hydr} for $M \cdot H_2O$ complexes derived from the slopes of van't Hoff plots are collected in table 2.

For doubly charged $(MH_2)^{2+}$ log J versus i_e plots and van't Hoff plots of the respective K_{ass} proved quite similar to those for singly charged ions, as illustrated for some selected species in fig. 7a and b. This is particularly well exemplified for the pair of $(MH_2)^{2+}$ (m/e 82.5) and $(MH \cdot H_2O)^+$ (m/e 182) of $m_2^{6,9}$Ade, originating from the same common parent hydrate $M(H_2O)_2$. For both ions log $J = f(i_e)$ (fig. 7a) and log $K_{ass} = f(T^{-1})$ (fig. 7b, curves 2 and 4) temperature functions take the same form and the enthalpies of hydration derived from the slopes of curves 2 and 4 proved to be the same within experimental error. Actually, $\Delta H_{hydr} = 29.5$ kJ mol^{-1} shown in table 2 for the reaction $m_2^{6,9}\text{Ade} \cdot H_2O + H_2O \leftrightharpoons m_2^{6,9}\text{Ade}(H_2O)_2$ is the average value of the two enthalpies.

In order to check whether any electric field contributions are included in the ΔH_{hydr} mass-spectrometric data, log $J = f(i_e)$ functions were recorded at various electric field strengths F_e in the reaction zone. However, no measurable effects were observed. This is in agreement with our earlier observations [10] made in connection with field-ionization mass spectrometric studies on A-U and A-T base-pair formation, and with recent theoretical calculations of interaction energies in such complexes under high electric field conditions [16]. Also, mass-spectrometric enthalpies of formation of water dimers, trimers, etc. [11], are consistent with available experimental [17] and theoretical [18] data.

4. Discussion

According to the magnitude of the apparent equilibrium constants for the formation of mono-, di- and trihydrates at 293 K (table 1), N-methylated adenines can be divided into three groups comprising: (i) singly substituted m^7Ade and m^9Ade, (ii) doubly substituted $m_2^{6,6}$Ade and $m_2^{6,9}$Ade, and (iii) fully methylated $m_3^{6,6,9}$Ade. It is then immediately obvious that mono-methylated adenines exhibit the most favourable hydration properties, di-methylated derivatives less so, while hydration of fully N-methylated adenine is the least favourable. In view of the approximations assumed in evaluation of the hydration equilibrium constants, the differences in $K_{\rm ass}$ between the members of each of the first two groups should be considered in connection with the independently obtained enthalpy data, with the reasonable assumption that the hydration reaction is mainly enthalpy-driven.

The enthalpies of hydration, $\Delta H_{\rm hydr}$, also generally decrease with the number of methyl groups substituted when going from m^{7}Ade to m$_3^{6,6,9}$ Ade, in the order exhibited by the $K_{\rm ass}^{\rm M\cdot H_2O}$. Values of $\Delta H_{\rm hydr}$ therefore support the conclusion drawn from consideration of the equilibrium data that methylation decreases the binding affinity of the adenines towards water. Values of $K_{\rm ass}^{\rm M\cdot H_2O}$ and $\Delta H_{\rm hydr}$ taken together for each of the pairs of singly and doubly methylated bases indicate that there exist also small differences in the stability of the respective monohydrates: m^{7}Ade · H_2O > m^{9}Ade · H_2O and m$_2^{6,6}$Ade · H_2O > m$_2^{6,9}$Ade · H_2O.

Inspection of $\Delta H_{\rm hydr}$ data for formation of di- and trihydrates leads to the conclusion that binding of a subsequent water molecule by a given monohydrate is also energetically less favourable, while the third water molecule is bound by the dihydrates with about the same energy as the second one by monohydrates.

We shall now try to reconcile these findings with the existing quantum-mechanical schemes of adenine hydration [4,6,19,20]. Although absolute values of the binding energies, calculated for a single water molecule and the same hydration centres, depend somewhat on the form of the potential function approximating interactions between bases and water, all hydration schemes re-

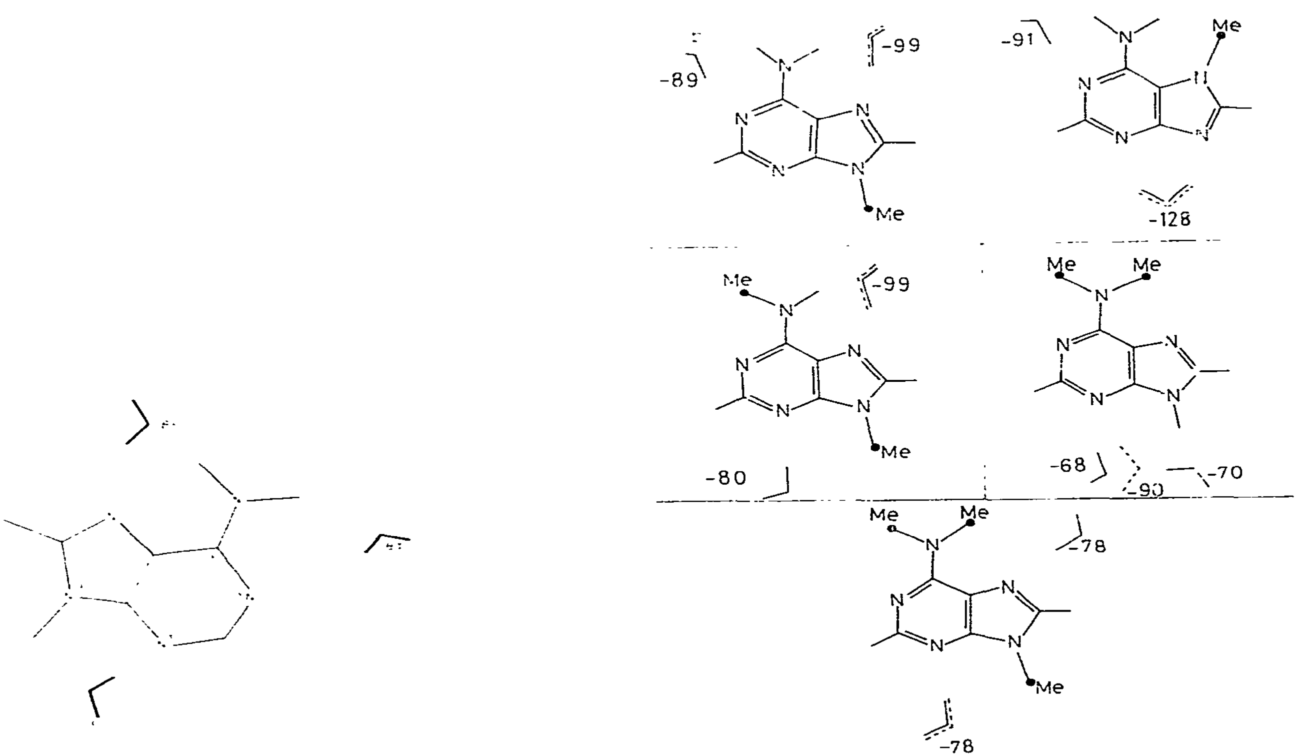

Fig. 8. Quantum-mechanical hydration schemes of adenine and of its N-methylated derivatives: (a) Energetically most favourable positions of water molecules located in the molecular plane of adenine [6]; −8.1, −8.6 and −8.9 are interaction energies in kcal/mol. (b) Most favourable positions of singly (⊰) and doubly (<) hydrated N-methyl adenines [20]; the figures correspond to interaction energies given in kcal/mol.

main in qualitative agreement. They are characterized by the presence of three deep minima of interaction energy located in the plane of the purine ring (cf. scheme in fig. 8a) between the two ring nitrogens N(9) and N(3), and between either of the two other ring nitrogens N(7) and N(1) and the exocyclic amino group nitrogen at C(6). It is obvious then that single methyl substitution at N(7) or N(9) in each case leaves the two strong hydration centres unperturbed. One would expect, therefore, a similar behaviour of the two bases in the monohydration reaction, in good agreement with the experimental data. Somewhat higher enthalpy and $K_{ass}^{M \cdot H_2O}$ of m^{7}Ade hydration than those of m^{9}Ade support the theoretically predicted [20] substantial increase in the binding energy of a water molecule at the N(3), N(9) site upon N(7) methyl substitution. According to this scheme, binding of water to m^{9}Ade takes place at the N(7), N(amino) site.

In both dimethylated adenines only one strong binding centre is left fully available for the monohydration reaction: the strongest one N(7), N(amino) in the case of m$_2^{6,9}$Ade (of the two rotational isomers possible for this compound, the one with aminomethyl group *trans* with respect to the imidazole N(7) nitrogen is expected to be thermodynamically more stable) and a weaker one at N(9). N(8) in that of m$_2^{6,6}$Ade. The order of the experimental hydration enthalpies and calculated binding energies is now, however, reversed. This point should thus deserve further studies.

Finally, in the case of the fully N-methylated derivative, viz., m$_3^{6,6,9}$Ade, all three hydrophilic binding centres of the adenine ring are less accessible to water because of the steric repulsion exerted thereon by the bulky methyl substituents at the amino and N(9) ring nitrogens. This is manifested in further lowering of both the equilibrium and energy parameters of monohydration of m$_3^{6,6,9}$Ade relative to those of di-*N*-methylated derivatives.

The experimental data for addition of the second and third water molecules to M · H_2O complexes are generally also explicable in terms of the theoretical hydration scheme of adenine, and of its *N*-methylated derivatives [20], in particular. According to the latter scheme (fig. 8b), binding of the second water molecule by monohydrates, except for m$_2^{6,6}$Ade · H_2O, does not perturb the configuration and the binding energy of the already bound water molecule. Only in the case of m^{9}Ade · H_2O is the weakest of the three hydrophilic hydration sites of adenine left fully accessible to the second water molecule. In all other instances binding of water takes place at more distant sites, characterized necessarily by less negative interaction energies (cf. scheme in fig. 8b). Thus, experimentally observed lowering of $K_{ass}^{M(H_2O)_2}$ and $K_{ass}^{M(H_2O)_3}$, as well as of the respective hydration enthalpies ΔH_{hydr} in the order m^{9}Ade > m$_2^{6,9}$Ade > m$_3^{6,6,9}$Ade is fully understandable. The unexpectedly lower values of $K_{ass}^{M(H_2O)_2}$ found for m^{7}Ade and m$_2^{6,6}$Ade can be due to higher ionization potentials of these two bases [12], because ionization coefficients of the respective $(MH \cdot H_2O)^+$, included in the values of the apparent association constants, may differ considerably from those of the other *N*-methylated adenines. In view of the theoretically predicted [20] substantial gain in the binding energy of water molecules in the m$_2^{6,6}$Ade · $2H_2O$ dihydrate, resulting from direct interaction of the two water molecules located close to the N(3) and N(9) ring nitrogen atoms (cf. scheme in fig. 8b), it would be of great interest to measure the enthalpy of this hydration reaction experimentally.

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