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Self-association of adenosine 5′-monophosphate (5′-AMP) as a function of pH and in comparison with adenosine, 2′-AMP and 3′-AMP

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The concentration dependence of the chemical shifts for protons H-2, H-8, and H-1′ of adenosine (Ado), 2′-AMP, 3′-AMP and 5′-AMP was measured in D_2O at 27 °C under several degrees of protonation. All results are consistent with the isodesmic model of indefinite noncooperative stacking. The association constants for Ado decrease with increasing protonation: Ado ($K = 15\ M^{-1}$) > $D(Ado)^+$/Ado ($6.0\ M^{-1}$) > $D(Ado)^+$ ($0.9\ M^{-1}$). In contrast, a maximum is observed with 5′-AMP: $5'\text{-}AMP^{2-}$ ($K = 2.1\ M^{-1}$) < $D(5'\text{-}AMP)^-$ ($3.4\ M^{-1}$) < $D_2(5'\text{-}AMP)^{\pm}$/$D(5'\text{-}AMP)^-$ ($5.6\ M^{-1}$) > $D_2(5'-AMP)^{\pm}$ ($\sim 2\ M^{-1}$) > $D_3(5'-AMP)^+$ ($\leqslant 1\ M^{-1}$). Self-stacking is most pronounced here if 50% of the adenine residues are protonated at N-1; complete base protonation reduces the stacking tendency drastically. Comparing the self-association of 2′-, 3′- and 5′-AMP shows that there is no influence of the phosphate-group position in the 2-fold negatively charged species, i.e., $K \simeq 2\ M^{-1}$ for all three AMP^{2-} species. More importantly, there is also no significant influence observed if the stacking tendency of the three $D_2(AMP)^+$/$D(AMP)^-$ 1:1 mixtures is compared ($K \simeq 6$–$7\ M^{-1}$); moreover, the measured association constants are within experimental error identical with the constant determined for $D(Ado)^+$/Ado ($K = 6.0\ M^{-1}$). This indicates that any coulombic contribution between the $-PO_3(H)^-$ group and the H^+ (N-1) unit of the adenine residue to the stability of the mentioned stacks in D_2O is small. However, experiments in 50% (v/v) dioxane-D_8/D_2O with the $D_2(5'\text{-}AMP)^{\pm}$/$D(5'\text{-}AMP)^-$ 1:1 system reveal, despite its low solubility, that coulombic interactions contribute to the self-association in an environment with a reduced polarity (compared to that of water). The implications of these observations for biological systems are briefly indicated.

1. Introduction

About one-sixth of all enzyme systems need cofactors which carry an adenine residue [1]. Prominent representatives of these cofactors are the adenine nucleotides and for these it was shown that self-association via base stacking is an important property [2–5]. It is now generally accepted [3–6] that their self-association occurs beyond the dimer stage and that oligomers are formed. 'Head-to-tail' stacking, with the five-membered and six-membered rings alternating in the stack, has been suggested [7], but other geometries are also possible [3,8–10].

Since enzyme-catalyzed reactions occur in active-site cavities which have a lower equivalent solution (or effective) dielectric constant than bulk water [11–13], the acid-base properties of monomeric adenosine 5′-monophosphate (5′-AMP; cf. fig. 1 [14,15]) have recently been studied in 50% aqueous dioxane [15], a solvent having a reduced polarity compared with water. Lowering of the

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Abbreviations and definitions: A, adenine derivative; Ado, adenosine; 2′-, 3′-, or 5′-AMP and 5′-ATP, adenosine 2′-, 3′- or 5′-mono- and 5′-triphosphate. The expression 'protonation' is used throughout this study for the addition of H^+ or D^+ to a basic site, i.e., independent from the kind of hydrogen isotope. However, the isotope which is used in a certain experiment or considered in a given equilibrium is always clearly defined.

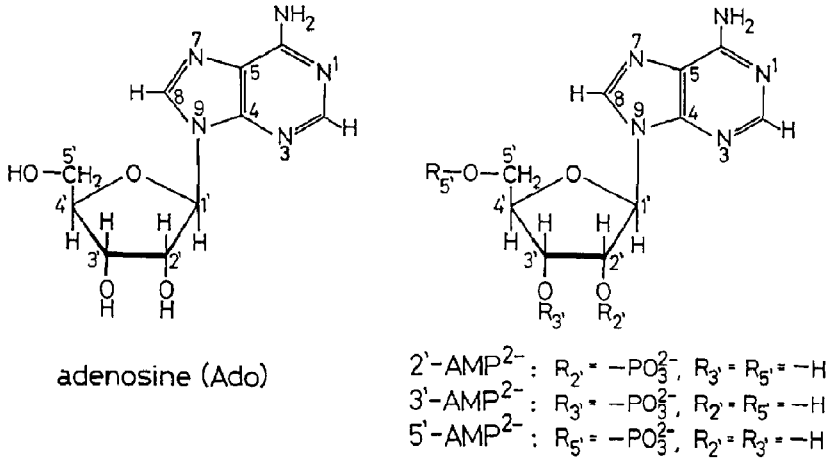

Fig. 1. Chemical structure of the compounds used in this study: adenosine and its nucleotide derivatives are shown in their dominating *anti* conformation [14,15].

solvent polarity facilitates removal of the proton from the H^+(N-1) site while the $-PO_3^{2-}$ group becomes more basic [15]; this increases the pH range over which the monoprotonated H(5′-AMP)$^-$ species is stable to the physiological pH region. This observation prompted us to study the self-association properties of 5′-AMP as a function of pH.

^{1}H-NMR shift measurements are ideal for studying the self-association of nucleotides [4,5] and the experiments carried out now reveal that the influence of pH on the self-stacking tendency of 5′-AMP is considerable: there is a promotional effect which reaches a maximum at that pH where the concentrations of H_2(AMP)$^\pm$ and H(AMP)$^-$ are equal to each other. This result contrasts with the adenosine system, in which any protonation leads to a decreased stacking tendency. With the aim of identifying possible ionic interactions within such stacks, 2′-AMP and 3′-AMP (fig. 1) were included in the study, thus allowing in combination with 5′-AMP an evaluation of the influence of the phosphate residue on the stability of the stacks.

2. Experimental

2.1. Materials

The adenosine monophosphates and all other reagents were from the same source as that recently described [15].

2.2. Apparatus and measurements

The ^{1}H-NMR spectra were recorded with a Bruker WH-90 FT spectrometer (90.025 MHz) at 27°C in D_2O or 50% (v/v) dioxane-D_8/D_2O as solvents, using the center peak of the tetramethylammonium ion triplet as internal reference (2.5×10^{-3} M) (for details and justification see ref. 4). However, all measured chemical shifts were converted to the sodium 3-(trimethylsilyl)propanesulfonate reference by adding 3.174 ppm (D_2O) [16] or 3.152 ppm (50% dioxane). To prevent interactions with paramagnetic metal impurities and to obtain sharp resonance signals 1% EDTA, based on the concentration of the AMP, was added to the solutions used in the experiments to study the self-association (see also, e.g., ref. 3). In the experiments with adenosine no EDTA was added.

The p*D* of the solutions was measured with a Metrohm EA 125 glass electrode connected with a Metrohm 654 digital pH meter (Metrohm AG, Herisau, Switzerland); the final p*D* of the D_2O solutions was obtained by adding 0.40 to the pH meter reading [17] (see also the comments in ref. 15 and in footnote 11 of ref. 18). The p*D* in 50% (v/v) dioxane-D_8/D_2O was determined in the same way; no further 'correction' was applied for the change in solvent, although correction factors have been published [19] for the change from

water to aqueous dioxane. The desired pD of a solution was adjusted by dotting with relatively concentrated NaOD or DNO_3 on a thin glass rod.

The ^{1}H-NMR signals of adenosine and its phosphates were assigned as described previously [4,5]. The concentration ranges in the D_2O solutions used in the experiments for quantification of the self-association at the various pD values were as follows: 5′-AMP at pD 8.90 (0.0078–0.40 M), pD 6.82 (0.0026–0.40 M), pD 5.61 (0.0024–0.40 M), pD 4.37 (0.005–0.40 M), pD 3.44 (0.0033–0.12 M) and pD 1.42 (0.004–0.11 M); 3′-AMP at pD 8.89 (0.0048–0.39 M) and pD 4.10 (0.0025–0.04 M); 2′-AMP at pD 8.87 (0.005–0.40 M) and 4.26 (0.005–0.20 M); adenosine at pD 7.0 (0.0025–0.051 M), pD 4.14 (0.0038–0.09 M) and pD 2.40 (0.0049–0.37 M). When the upper concentration limit is lower than 0.4 M, the given concentration is close to the solubility limit. The ionic strength (I) was adjusted with $NaNO_3$ to 0.1 M when necessary; however, in several cases I varied between 0.1 M and much larger values (see tables).

2.3. Calculations

All experimental data were analyzed with a Hewlett-Packard 9825A calculator connected to a Hewlett-Packard 7470A plotter and a Hewlett-Packard 82905B printer by using a Newton-Gauss nonlinear least-squares method. The results were calculated with a curve-fitting program based on the isodesmic model of indefinite noncooperative self-association (see eqs. 2 and 3). Adaption of this model to ^{1}H-NMR shift measurements [2,4,20] led to the relationship

$$\delta_{obsd} = \delta_\infty + (\delta_\infty - \delta_0)\left[1 - (4K[A] + 1)^{1/2}\right] / 2K[A] \quad (1)$$

for the observed chemical shift (δ_{obsd}) and the total concentration [A]; δ_0 represents the shift at infinite dilution (monomeric A) and δ_∞ the shift of a molecule in an infinitely long stack, while K is the association constant as defined in eq. 2 (vide infra). By ignoring species larger than dimers, a relationship similar to eq. 1 is obtained, but δ_∞ is replaced by δ_D, the upfield shift in a dimer, and K is replaced by $2K_D$ (i.e., $K_D = 0.5K$), which is the equilibrium constant for dimerization [2,4,20].

The reduced solubility of 5′-AMP in acidic solution provided a special problem: The measured data in the right-hand part of fig. 3 (see section 3) show that the concentration range employed at pD 1.42 and 3.44 is much smaller, i.e., only 0.004–0.12 M, than that in the other cases, where the range could be extended to 0.4 M. For the usual curve fitting with its iteration procedure this small range is not sufficient; the 'bend' of the data curve is too insignificant to allow the determination of three variables (eq. 1). However, evaluation of the experiments at p$D \geq 4.37$ was straightforward (tables 1 and 2) and from the first four entries in table 2 (vide infra) it is evident that $\Delta\delta$ ($= \delta_0 - \delta_\infty$) for H-8 and H-1′ is independent of pD within experimental error. Therefore the values of $\Delta\delta$ for each of these two protons were averaged and the resulting values used in the calculations. The value for $\Delta\delta$ of H-2 is more difficult to estimate, because of its dependence on pD (table 2). Fig. 2 shows the computer-calculated best fit through the measured data by taking into account the known acidity constants for $D_2(AMP)^{\pm}$ [15]; interpolation at pD 3.44 and 1.42 gives estimates of the corresponding $\Delta\delta_{H\text{-}2}$ values. Now, with values for $\Delta\delta_{H\text{-}2}$, $\Delta\delta_{H\text{-}8}$ and $\Delta\delta_{H\text{-}1'}$ the experimental data at pD 1.42 and 3.44 could easily

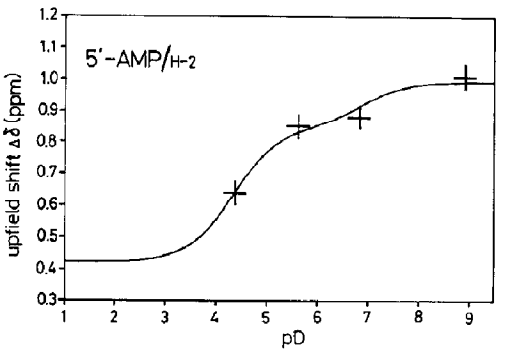

Fig. 2. Dependence of the upfield shift $\Delta\delta$ on pD for H-2 of self-stacked 5′-AMP. The computer-calculated best fit of the experimental data at pD 8.90, 6.82, 5.61 and 4.37 by taking into account $pK^D_{D(5'\text{-AMP})} = 6.83$ and $pK^D_{D_2(5'\text{-AMP})} = 4.33$ (from ref. 15) allows extrapolation of the values for $\Delta\delta$ at pD 3.44 and 1.42 (see table 2). The vertical error bars correspond to ± 0.04 ppm. The estimates are $\Delta\delta_{(3.44)} = 0.49$ ppm and $\Delta\delta_{(1.42)} = 0.45$ ppm.

be evaluated: with only two unknowns (K and δ_0; see eq. 1), δ_∞ being fixed due to $\delta_\infty = \delta_0 - \Delta\delta$, the fitting procedure converged easily.

3. Results and discussion

Self-association of nucleotides is ideally studied by ^{1}H-NMR shift measurements [4,5]: upfield shifts observed with increasing concentration of the nucleotides confirm stack formation and allow a quantitative evaluation of its extent by employing the isodesmic model for indefinite noncooperative self-association [4,5,21,22]. This model is based on the assumption that, e.g., for an adenine derivative (A), the equilibrium constants (eq. 2) for the equilibria (eq. 3) are all equal:

$$K = [(A)_{n+1}]/[(A)_n][A] \quad (2)$$

$$(A)_n + A \rightleftharpoons (A)_{n+1} \quad (3)$$

Indeed, all the observations made now in ^{1}H-NMR shift experiments can be well described with eq. 2 (see also section 2). This agrees with previous results [4,5,15,23,24] for other purine nucleotides.

3.1. Influence of the protonation degree on the self-stacking tendency of 5′-AMP

Three representative ^{1}H-NMR shift experiments carried out with 5′-AMP in D_2O as solvent are shown in fig. 3: the variation of the upfield shifts for H-2, H-8 and H-1′ (fig. 1) as a function of the 5′-AMP concentration is clearly seen. The 'curvature' of the experimental points is most pronounced at pD 4.37; hence, without any mathematical evaluation it is immediately apparent that self-stacking is more pronounced at the intermediate pD 4.37 than at pD 1.42 and 8.90. Application of the isodesmic model for an indefinite noncooperative self-association (eqs. 1–3) leads to the solid curves shown in fig. 3. In this connection it should be emphasized that application of any model introducing one or more additional variables will also be able to explain the experimental data. For example, previously, reasonings have been presented: (i) for the application of a two-constant attenuated equilibrium model [25] and there are indications that the isodesmic model somewhat overpredicts the amount of higher polymers [25]; (ii) for the consideration of the chemical shifts due to dimers and trimers [26] (see also ref. 2); or (iii) for the consideration of the thermodynamic nonideality of the monomer and polymers [6,27]. Hence, it is evident that the applied isodesmic model (eqs. 1–3) is a 'limiting' model, which describes the experimental situation well (fig. 3) and therefore the consideration of further variables is not justified; however, this also means that the actual situation could well be more complicated but not simpler. Clearly, alterations

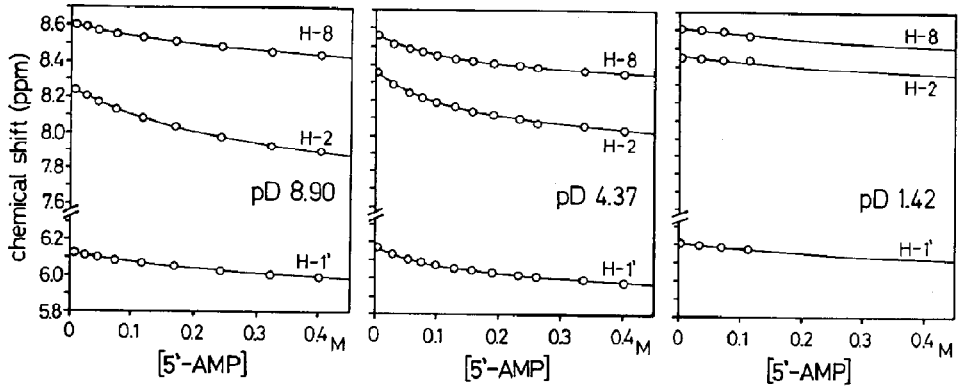

Fig. 3. Variation of the chemical shift of H-2, H-8 and H-1′ with increasing concentrations of 5′-AMP at pD 8.90 (cf. footnote c in table 1), 4.37 and 1.42 (from left to right). The spectra were measured on a Bruker FT 90 at 90.025 MHz (D_2O; 27 °C; for I see table 1), relative to internal $(CH_3)_4N^+$ and converted to values relative to sodium 3-(trimethylsilyl) propanesulfonate by adding 3.174 ppm. The curves shown here are the computer-calculated best fit of the experimental data (calculated with K_{av} of table 1), using the indefinite noncooperative stacking model (eqs. 1 and 2); the resulting shifts are listed in table 2.

Table 1

Association constants for self-stacking (eq. 2) of 5′-AMP, its 2′ and 3′-isomers, and adenosine at various pD values in D_2O solutions as determined by ^{1}H-NMR shift measurements [a] at 27 °C

System	pD	Species [b] mainly present	Approximate concentration of species [b] (%)	I (M)	K (M^{-1}) determined from the shift of			K_{av} (M^{-1})
					H-2	H-8	H-1′	
5′-AMP	8.90 [c]	5′-AMP^{2-}	100	0.1– ~ 1.2	2.32 ± 0.21	1.77 ± 0.27	1.80 ± 0.36	2.1 ± 0.3
	6.82 [d]	AMP^{2-}/D(AMP)$^-$	50/50	0.1– ~ 0.8	3.07 ± 0.20	3.44 ± 0.39	2.53 ± 0.18	2.9 ± 0.3
	5.61	D(AMP)$^-$	90	0.1– ~ 0.6	3.74 ± 0.27	3.40 ± 0.29	3.07 ± 0.28	3.4 ± 0.3
	4.37 [e]	D(AMP)$^-$/D$_2$(AMP)$^\pm$	50/50	0.1– ~ 0.6	5.95 ± 0.60	5.35 ± 0.55	5.57 ± 0.33	5.6 ± 0.5
	3.44 [f]	D$_2$(AMP)$^\pm$/D(AMP)$^-$	90/10 [f]	0.1– ~ 0.2	2.07 ± 0.63	1.91 ± 0.39	2.48 ± 0.56	2.1 ± 0.6
	1.42 [f,g]	D$_2$(AMP)$^\pm$/D$_3$(AMP)$^+$	75/25 [f]	0.1– ~ 0.2	0.31 ± 0.13	0.98 ± 0.34	0.96 ± 0.13	0.9 ± 0.2
Ado	7.00 [c]	Ado	100	0.1	13.7 ± 3.4	18.7 ± 5.4	15.1 ± 4.9	15 ± 3
	4.14 [h]	Ado/D(Ado)$^+$	50/50	0.1	5.82 ± 0.98	6.64 ± 1.66	6.01 ± 0.96	6.0 ± 1.3
	2.40	D(Ado)$^+$	100	0.1– ~ 0.4	0.80 ± 0.33	0.57 ± 0.16	0.99 ± 0.14	0.9 ± 0.2
2′-AMP	8.87 [i]	2′-AMP^{2-}	100	0.1– ~ 1.2	2.06 ± 0.09	2.07 ± 0.20	1.63 ± 0.21	2.0 ± 0.2
	4.26 [j,k]	D(AMP)$^-$/D$_2$(AMP)$^\pm$	50/50	0.1– ~ 0.2	8.94 ± 1.36	3.73 ± 1.54	7.36 ± 2.14	7.9 ± 2.0
3′-AMP	8.89 [i]	3′-AMP^{2-}	100	0.1– ~ 1.2	2.08 ± 0.18	1.56 ± 0.13	1.03 ± 0.13	1.6 ± 0.4
	4.10 [k,l]	D(AMP)$^-$/D$_2$(AMP)$^\pm$	50/50	0.1	6.96 ± 3.62	12.02 ± 9.26	5.35 ± 2.71	7 ± 4

[a] The results were obtained by evaluating experiments like the examples shown in fig. 3; for the concentration ranges employed see section 2. The ionic strength (I) was adjusted to 0.1 by adding $NaNO_3$ where necessary. The range of error given with the values for K of the individual protons corresponds to the standard deviation (σ). K_{av} is the weighted mean of the individual results calculated via log K; the range of error given here is twice the standard error (2σ).

[b] This refers to the protonation degree only (for the extent of stacking see, e.g., fig. 5); the given percentages are based on the acidity constants given in the text in eqs. 4–9.

[c] These data are the results of reevaluations of earlier experiments (5′-AMP [4,15], adenosine [2]); formerly for $(CH_3)_4N^+$ the reference shift $\delta_{TMA} = 3.188$ was taken, while now the value 3.174 ppm is used (see section 2).

[d] $pD \simeq pK^D_{D(5'\text{-AMP})} = 6.83$ [15].

[e] $pD = pK^D_{D_2(5'\text{-AMP})} = 4.33$ [15].

[f] See footnote b of table 2.

[g] Complete protonation of the phosphate group of 5′-AMP in D_2O gives the species $D_3(AMP)^+$: $pK^D_{D_3(AMP)} = 0.9$ [15]. The N-7 site of 5′-AMP is not protonated under these conditions; its affinity for H^+ is very low: $pK_a = -1.6$ [30].

[h] $pD = pK^D_{D(Ado)} = 4.14$ [15].

[i] From ref. 15.

[j] $pD \simeq pK^D_{D_2(2'\text{-AMP})} = 4.23$ [15].

[k] Measurements at lower pD, e.g., $pD = 3.2 \simeq pK^D_{D_2(AMP)} - 1$, were not possible due to the low solubility of 2′-AMP (< 0.03 M) and 3′-AMP (< 0.02 M)

[l] $pD = pK^D_{D_2(3'\text{-AMP})} = 4.10$ [15].

of the ionic strength [28] or the presence of other ions, such as Mg^{2+} [4,5,28], are expected to affect the extent of the self-association via stacking [2–10]; no contribution to the self-association via hydrogen bonding has been found for 5′-AMP in aqueous solution [29].

The results obtained from the isodesmic-model evaluation, i.e., the association constants (eqs. 2 and 3) quantifying the stack formation and the corresponding alterations of the chemical shifts, are summarized in tables 1 and 2 [30], respectively. In table 1 are also given the monomeric species mainly present at the pD of an experiment together with their approximate concentrations. These results for 5′-AMP are based on the following equilibria, for which the acidity constants in D_2O are known from ref. 15:

$$D_3(5'\text{-AMP})^+ \rightleftharpoons D_2(5'\text{-AMP})^\pm + D^+ \quad (4a)$$

$$K^D_{D_3(5'\text{-AMP})} = \left[D_2(5'\text{-AMP})^\pm\right][D^+]/\left[D_3(5'\text{-AMP})^+\right] = 10^{-0.9\pm0.2} \quad (4b)$$

Table 2

Chemical shifts (ppm) of the protons for monomeric (δ_0) and self-stacked (δ_∞) 5′-AMP, its 2′- and 3′-isomers, and adenosine at various pD values in D_2O solutions, together with the corresponding upfield shifts ($\Delta\delta = \delta_0 - \delta_\infty$) [a]

System [a]	pD [a]	H-2			H-8			H-1′		
		δ_0	δ_∞	$\Delta\delta$	δ_0	δ_∞	$\Delta\delta$	δ_0	δ_∞	$\Delta\delta$
5′-AMP	8.90 [c]	8.251 ± 0.009	7.24 ± 0.09	1.01 ± 0.09	8.608 ± 0.005	8.15 ± 0.04	0.46 ± 0.04	6.131 ± 0.005	5.75 ± 0.04	0.38 ± 0.04
	6.82	8.254 ± 0.007	7.37 ± 0.05	0.88 ± 0.05	8.537 ± 0.005	8.12 ± 0.03	0.42 ± 0.03	6.134 ± 0.004	5.74 ± 0.02	0.39 ± 0.02
	5.61	8.258 ± 0.008	7.41 ± 0.04	0.85 ± 0.04	8.482 ± 0.004	8.04 ± 0.02	0.44 ± 0.02	6.137 ± 0.004	5.71 ± 0.02	0.43 ± 0.02
	4.37	8.370 ± 0.011	7.73 ± 0.03	0.64 ± 0.03	8.569 ± 0.007	8.16 ± 0.02	0.41 ± 0.02	6.178 ± 0.005	5.81 ± 0.02	0.37 ± 0.02
	3.44 [b]	8.431 ± 0.014	7.96 [b]	0.47 ± 0.10 [b]	8.624 ± 0.013	8.19 [b]	0.43 ± 0.05 [b]	6.199 ± 0.012	5.81 [b]	0.39 ± 0.05 [b]
	1.42 [b]	8.461 ± 0.013	8.04 [b]	0.42 ± 0.10 [b]	8.609 ± 0.010	8.18 [b]	0.43 ± 0.05 [b]	6.207 ± 0.010	5.82 [b]	0.39 ± 0.05 [b]
Ado	7.00 [c]	8.264 ± 0.009	7.76 ± 0.07	0.50 ± 0.07	8.336 ± 0.006	8.06 ± 0.04	0.28 ± 0.04	6.073 ± 0.005	5.85 ± 0.04	0.22 ± 0.04
	4.14	8.364 ± 0.007	7.71 ± 0.10	0.65 ± 0.10	8.445 ± 0.006	8.02 ± 0.07	0.42 ± 0.07	6.119 ± 0.004	5.78 ± 0.05	0.34 ± 0.05
	2.40	8.452 ± 0.003	8.15 ± 0.06	0.30 ± 0.06	8.545 ± 0.002	8.21 ± 0.06	0.34 ± 0.06	6.160 ± 0.002	5.77 ± 0.07	0.39 ± 0.07
2′-AMP	8.87 [d]	8.255 ± 0.005	7.46 ± 0.05	0.80 ± 0.05	8.369 ± 0.002	8.09 ± 0.02	0.28 ± 0.02	6.151 ± 0.002	6.00 ± 0.01	0.15 ± 0.01
	4.26 [c]	8.361 ± 0.019	7.71 ± 0.08	0.65 ± 0.09	8.443 ± 0.011	8.24 ± 0.04	0.20 ± 0.04	6.242 ± 0.006	6.08 ± 0.02	0.16 ± 0.02
3′-AMP	8.89 [d]	8.248 ± 0.011	7.05 ± 0.19	1.20 ± 0.19	8.368 ± 0.005	7.79 ± 0.09	0.58 ± 0.09	6.108 ± 0.004	5.69 ± 0.07	0.42 ± 0.07
	4.10 [e]	8.379 ± 0.007	7.92 ± 0.22	0.46 ± 0.22	8.482 ± 0.006	8.18 ± 0.16	0.30 ± 0.16	6.169 ± 0.004	5.90 ± 0.13	0.27 ± 0.13

[a] Experimental conditions are the same as those listed in table 1. The chemical shifts were measured relative to internal $(CH_3)_4N^+$ and converted to values downfield from sodium 3-(trimethylsilyl)propanesulfonate by adding 3.174 ppm. The shifts were calculated by using the values of K_{av} together with their corresponding errors in table 1; the range of error given with the calculated shifts is twice the standard deviation. [b]

[b] Due to reduced solubility, the concentration range was too small (0.004 to ~ 0.1 M; see fig. 3) for the usual curve fitting with its iteration procedure. Therefore, values for $\Delta\delta$ were estimated as described in section 2.3. The error limits for the values of $\Delta\delta$ are also estimates and represent rather upper limits. K was then calculated with the known $\Delta\delta$, the error being based on the estimated error of $\Delta\delta$; K_{av} was determined as described in table 1. The final values for δ_0 were obtained from a curve fitting with the known values for $\Delta\delta$ and K_{av}; δ_∞ is equal to $\delta_0 - \Delta\delta$.

[c] See footnote c of table 1.

[d] From ref. 15.

[e] See footnote k of table 1.

$$D_2(5'\text{-AMP})^{\pm} \rightleftharpoons D(5'\text{-AMP})^{-} + D^{+} \quad (5a)$$

$$K^{D}_{D_2(5'\text{-AMP})} = [D(5'\text{-AMP})^{-}][D^{+}]/[D_2(5'\text{-AMP})^{\pm}] = 10^{-4.33 \pm 0.02} \quad (5b)$$

$$D(5'\text{-AMP})^{-} \rightleftharpoons 5'\text{-AMP}^{2-} + D^{+} \quad (6a)$$

$$K^{D}_{D(5'\text{-AMP})} = [5'\text{-AMP}^{2-}][D^{+}]/[D(5'\text{-AMP})^{-}] = 10^{-6.83 \pm 0.04} \quad (6b)$$

The release of the first proton from $D_3(5'\text{-AMP})^{+}$ occurs from the phosphate group (eq. 4), resulting in a negatively charged phosphate residue leading thus to the zwitterionic species $D_2(5'\text{-AMP})^{\pm}$; the base proton at N-1 in the species is released next (eq. 5), giving the anion $D(5'\text{-AMP})^{-}$, which in the final step (eq. 6) loses its last proton from the phosphate group forming $5'\text{-AMP}^{2-}$.

It is evident that the stacks at a given p*D* are mainly composed of the monomeric species dominating at that p*D*. One might argue that with increasing stacking the acid/base properties valid for the monomeric species are altered due to coulombic interactions in the stacks, so that the above acidity constants are changed; to some degree this might be true. However, these alterations seem to be small, or in any case do not significantly influence the chemical shifts: at a given p*D* the computer-calculated best fit of the experimental data based on eq. 1 is excellent in all cases over the whole concentration range (up to 0.4 M) (see, e.g., the two left-hand parts in fig.3). Indeed, this conclusion agrees with two other observations: (i) comparisons with the adenosine system indicate that ionic interactions stabilizing the stacks are not very important (see section 3.2); and (ii) in the concentration range studied the main stacks are the dimer, trimer and tetramer (see fig. 5); in other words, these associations are too small to exert significant inter-ionic charge effects as they occur in certain highly charged polymers.

The stacking interaction is most pronounced at p*D* 4.37 (table 1), i.e., under conditions where all nucleotide species carry a single proton at the phosphate group and where in addition about every second nucleotide is also protonated at N-1 giving thus a 1:1 mixture of $D(5'\text{-AMP})^{-}$ and $D_2(5'\text{-AMP})^{\pm}$ (eq. 5). That this partial protonation at N-1 is crucial for the increased stability of the stacks is evident from a comparison of the results obtained for p*D* 3.44 (cf. eq. 5) and 1.42 (cf. eq. 4) (table 1): further protonation at N-1 decreases the stability of the stacks. This is obviously due to repulsion between the positive charges located at the aromatic-ring systems; a corresponding observation has recently been made [24] with the self-association of $Zn(\epsilon\text{-ATP})^{2-}$. On the other hand, comparison of the results at p*D* 8.90, 6.82 and 5.61 indicates that addition of one proton to the phosphate group favors stacking due to charge neutralization; i.e., $D(5'\text{-AMP})^{-}$ self-stacks better ($K = 3.4\ M^{-1}$) than $5'\text{-AMP}^{2-}$ ($K = 2.1\ M^{-1}$). This result fits well into previous observations [5] leading overall to the series: $5'\text{-ATP}^{4-}$ ($K = 1.3\ M^{-1}$) < $5'\text{-ADP}^{3-}$ ($1.8\ M^{-1}$) < $5'\text{-AMP}^{2-}$ ($2.1\ M^{-1}$) < $D(5'\text{-AMP})^{-}$ ($3.4\ M^{-1}$) < Ado ($15\ M^{-1}$).

It is interesting to view with these results in mind the chemical shifts listed in table 2. The values determined for δ_0, i.e. for the monomeric unstacked species, agree well with those of a recent study [15] in which the chemical shift of H-2, H-8 and H-1′ as a function of p*D* was measured: the alteration of the shift of H-2 reflects mainly only the protonation at N-1, while H-8 is also sensitive to proton-phosphate group interactions. Hence, these observations correspond to the expectations. More surprising is the fact that the values of $\Delta\delta$ for H-8 and H-1′ remain practically unaltered from p*D* 1.4 to 8.9; this could be taken as an indication that the geometry of the stacks does not change much despite the different degrees of protonation over this pH range. However, $\Delta\delta$ of H-2 changes in a p*D*-dependent manner (see also fig. 2): this might mean that in a head-to-tail arrangement the single-fold charged phosphate group of one 5′-AMP approaches the protonated and positively charged N-1 site of the next 5′-AMP, allowing a weak ionic interaction. It is worth noting in this connection that the X-ray structure [31] of 5′-AMP indicates an intermolecular interaction between the phosphate oxygen of one 5′-AMP and the protonated N-1 unit of another 5′-AMP. However, the results given in

section 3.2 also indicate that this interaction must be weak in solution.

3.2. Self-association of adenosine as a function of pD and in comparison with the 5′-AMP system

To see how the 5′-phosphate group of AMP might influence stacking via an interaction with the positively charged H^+(N-1) site (at pD 0.9, about 50% of the 5′-AMP species still carry one negative charge at the phosphate residue; eq. 4), the self-stacking properties of adenosine were studied as a function of the protonation degree. The position of the acid/base equilibrium for adenosine in D_2O was recently determined [15].

$$D(Ado)^+ \rightleftharpoons Ado + D^+ \quad (7a)$$

$$K^D_{D(Ado)} = [Ado][D^+]/[D(Ado)^+] = 10^{-4.14 \pm 0.05} \quad (7b)$$

Hence, in D_2O solutions at pD 4.14 about 50% of adenosine is protonated at N-1, carrying a positive charge, whereas about 2 pH units above or below this value adenosine exists to an extent of nearly 100% as the free molecule or in its completely protonated form, respectively.

With this in mind, the pD values for the ^{1}H-NMR shift experiments were selected. The results are also listed in tables 1 and 2. The accessible concentration ranges for the measurements, especially at pD 7 (0.0025 to ~ 0.05 M) [2], are very small giving relatively large errors in K, δ_∞ and $\Delta\delta$, despite the rather pronounced self-association at pD 7.0 and 4.14. However, the self-association of Ado clearly decreases with increasing degree of protonation at N-1. This result corresponds with earlier observations [32,33] on purine bases and may be explained by postulating that increased base protonation decreases self-association due to repulsion.

To facilitate comparison of the stacking tendencies of Ado and 5′-AMP the corresponding association constants in table 1 are plotted with respect to pD in fig. 4. Two conclusions are evident: (i) At pD values where both adenine derivatives are fully protonated at N-1, the same stabilities of the stacks are observed. (ii) Much more surprising is that under conditions where 50% of

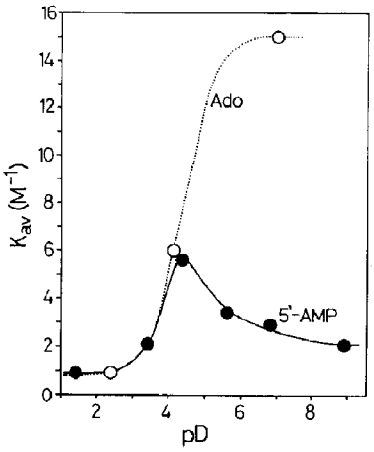

Fig. 4. Dependence of the self-association constant K_{av} (eq. 2) on pD for 5′-AMP (●) and adenosine (○) (see table 1). The course of the curves K_{av} (M^{-1}) vs. pD for adenosine or 5′-AMP should be compared with the corresponding acidity constants [15], $pK^D_{D(Ado)} = 4.14$ or $pK^D_{D(5'\text{-AMP})} = 6.83$ and $pK^D_{D_2(5'\text{-AMP})} = 4.33$.

the species are protonated at N-1 (pD = p$K_{a/H(N-1)}$) the self-stacking tendencies of the Ado and 5′-AMP systems are also identical within experimental error. Thus, one may conclude that the single-negatively charged phosphate residue in 5′-AMP has little influence on the self-association. The similarity of the stacks of Ado and 5′-AMP at pD = p$K_{a/H(N-1)}$ is confirmed by the $\Delta\delta$ values at pD < 4.4 (table 2) which are within experimental error identical for all three protons, i.e., H-2, H-8 and H-1′, of adenosine and 5′-AMP. At pD > 5 the situation is different, viz., the $\Delta\delta$ values of Ado and 5′-AMP^{2-} differ very strongly (table 2).

The results could mean (i) that two opposing effects, i.e., the favoring charge neutralization resulting from protonation at the phosphate group and the inhibiting protonation at the N-1 site, lead to the observed maximum (fig. 4) in the 5′-AMP system. This explanation agrees obviously without additional assumptions with the Ado system. However, the results could also mean (ii) that maximum self-association occurs at a 1 : 1 ratio of D_2(5′-AMP)$^{\pm}$/D(5′-AMP)$^-$ (table 1 and fig. 4), due to an especially pronounced interaction between the adenine moieties under these conditions. If the influence of negatively charged phos-

phate groups is ignored, we are left with stacks in which a positively charged base alternates with an uncharged aromatic-ring system. Donor-acceptor interactions should be especially favored in such a system.

The large stability of the uncharged adenosine stacks could then for case (ii) be explained by assuming that the reduced donor-acceptor interaction is overcompensated by an ideal orientation of the adenine residues allowing a maximum of dipole-dipole and hydrophobic-type interactions. The lower stability of the 5′-AMP^{2-} stacks would then be the result of simple charge repulsion; in agreement with this latter point is the fact that the stability of the AMP^{2-} stacks is independent of the position of the 2-fold negatively charged phosphate group, i.e., 2′-AMP^{2-}, 3′-AMP^{2-} and 5′-AMP^{2-} all have an association constant K close to 2 M^{-1} (see ref. 15).

In any case, the equal stacking tendency of Ado and 5′-AMP at $pD = pK_{a/H(N-1)}$ (fig. 4 and table 1) may be explained by the overall charges: in the $D_2(AMP)^{\pm}/D(AMP)^-$ system actually 'zero'/'minus' species interact with their +/0 charged aromatic-ring moieties, while in the $D(Ado)^+/Ado$ system again +/0 aromatic-ring interactions occur, the overall charge of the species also being +/0. Hence, the single negative charge at the phosphate group in $D_2(AMP)^{\pm}/D(AMP)^-$ is not reflected in a pronounced stack-promoting ionic interaction because one species carries the overall charge zero.

3.3. Comparison of the self-association of 2′-AMP, 3′-AMP and 5′-AMP

As indicated before, the position of the 2-fold charged phosphate group in AMP^{2-} does not significantly influence the stability of the corresponding stacks [15]. Similarly, under conditions where N-1 is fully protonated, for 2′-AMP and 3′-AMP repulsion between the aromatic ring systems is also expected and self-association should level off with $K \sim 0.9$ M^{-1} as observed for the D_3(5′-$AMP)^+$ and $D(Ado)^+$ systems (see table 1 and fig. 4). Unfortunately, the corresponding experiments could not be carried out due to the poor solubility of 2′-AMP and 3′-AMP at the required low pD values.

However, 2′-AMP and 3′-AMP in comparison with 5′-AMP still offer the possibility of checking whether any ionic contribution to the stability of the stacks in the $D_2(AMP)^{\pm}/D(AMP)^-$ systems is indeed negligible as indicated in sections 3.1 and 3.2. Therefore, further ^{1}H-NMR experiments were carried out in D_2O under conditions where N-1 in 2′-AMP and 3′-AMP is protonated to an extent of 50%. The acid/base equilibrium constants in D_2O for both nucleotides are known [15]:

$$D_2(AMP)^{\pm} \rightleftharpoons D(AMP)^- + D^+ \quad (8a)$$

$$K^D_{D_2(2'\text{-AMP})} = [D(2'\text{-AMP})^-][D^+]/[D_2(2'\text{-AMP})^{\pm}] = 10^{-4.23 \pm 0.03} \quad (8b)$$

$$K^D_{D_2(3'\text{-AMP})} = [D(3'\text{-AMP})^-][D^+]/[D_2(3'\text{-AMP})^{\pm}] = 10^{-4.10 \pm 0.04} \quad (8c)$$

$$D(AMP)^- \rightleftharpoons AMP^{2-} + D^+ \quad (9a)$$

$$K^D_{D(2'\text{-AMP})} = [2'\text{-}AMP^{2-}][D^+]/[D(2'\text{-AMP})^-] = 10^{-6.5} \quad (9b)$$

$$K^D_{D(3'-\text{AMP})} = [3'\text{-}AMP^{2-}][D^+]/[D(3'\text{-AMP})^-] = 10^{-6.3} \quad (9c)$$

Hence, the self-stacking properties of the 2′-AMP and 3′-AMP systems were then measured at pD about 4.23 and 4.10, respectively.

The association constants for stack formation of the D_2(2′-$AMP)^{\pm}/D$(2′-$AMP)^-$ and D_2(3′-$AMP)^{\pm}/D$(3′-$AMP)^-$ systems, each at a 1 : 1 ratio, are also listed in table 1 and the corresponding chemical shift data are given in table 2. The position of the phosphate group influences the stacking tendency only slightly, if at all: the stacking association constants (eq. 2) for the 1 : 1 $D_2(AMP)^{\pm}/D(AMP)^-$ systems are within experimental error identical for all three isomers of AMP. Thus, this result confirms the conclusions given in section 3.2: Coulombic interactions between the $-PO_3(H)^-$ moiety and the H^+(N-1) unit do not contribute significantly in D_2O to the

stability of the stacks formed between the $D(Ado)^+/Ado$ moieties.

3.4. Influence of dioxane at maximum stack formation of AMP

It is evident, that any weak ionic interaction stabilizing stacks would be favored in a solvent having a reduced polarity, compared with that of water, and hence the possible contribution of such an interaction should become more easily recognizable. Since 50% aqueous dioxane has a dielectric constant ($\epsilon = 35$) [11] similar to the 'equivalent solution' dielectric constant estimated for the active-site cavity of an enzyme [11], we carried out an experiment in 50% (v/v) dioxane-D_8/D_2O to determine how reduced polarity influences stack formation when 50% of the adenine moieties are protonated at N-1. We selected 5'-AMP because its acidity constants in this mixed solvent are known ($I = 0.1$, $NaNO_3$; 25 °C) [15]:

$$D_3(5'\text{-AMP})^+ \rightleftharpoons D_2(5'\text{-AMP})^\pm + D^+ \qquad (10a)$$

$$K^D_{D_3(5'\text{-AMP})} = \left[D_2(5'\text{-AMP})^\pm\right][D^+]/\left[D_3(5'\text{-AMP})^+\right] = 10^{-2.23 \pm 0.10} \qquad (10b)$$

$$D_2(5'\text{-AMP})^\pm \rightleftharpoons D(5'\text{-AMP})^- + D^+ \qquad (11a)$$

$$K^D_{D_2(5'\text{-AMP})} = \left[D(5'\text{-AMP})^-\right][D^+]/\left[D_2(5'\text{-AMP})^\pm\right] = 10^{-3.83 \pm 0.05} \qquad (11b)$$

$$D(5'\text{-AMP})^- \rightleftharpoons 5'\text{-AMP}^{2-} + D^+ \qquad (12a)$$

$$K^D_{D(5'\text{-AMP})} = [5'\text{-AMP}^{2-}][D^+]/[D(5'\text{-AMP})^-] = 10^{-8.06 \pm 0.06} \qquad (12b)$$

In accord with the above acidity constants an experiment was carried out at pD 3.83. However, the result was hampered by the low solubility of the $D_2(5'\text{-AMP})^\pm/D(5'\text{-AMP})^-$ 1:1 system in 50% (v/v) dioxane-D_8/D_2O ([5'-AMP] < 0.02 M) giving only a small concentration range for the measurements. Though clearcut upfield shifts were measured no significant saturation (i.e., a curvature analogous to that seen in fig. 3) could be reached, preventing the usual curve-fitting evaluation procedure. An evaluation of the data became possible only by using for H-2, H-8 and H-1' the values of $\Delta\delta$ determined in D_2O at pD 4.37 (see table 2); these values also correspond to a protonation degree of 50% at N-1. Consequently, the association constant, $K \simeq 3\ M^{-1}$ ($I = 0.1$, $NaNO_3$; 27 °C), as calculated for the self-association of the $D_2(5'\text{-AMP})^\pm/D(AMP)^-$ 1:1 system in 50% (v/v) dioxane-D_8/D_2O, can only be considered as a rough estimate.

Nevertheless, the following conclusion is still possible: Compared with the situation in D_2O ($K = 5.6 \pm 0.5\ M^{-1}$; table 1) the stacking tendency decreases by a factor of about 1/2 ($K \sim 3\ M^{-1}$). This must be compared with systems allowing only aromatic-ring stacking: for 2,2'-bipyridyl and 1,10-phenanthroline the stacking tendency decreases by changing the solvent from D_2O to 50% (v/v) dioxane-D_8/D_2O by factors of about 1/20 and 1/50, respectively [16]. This indicates that the self-association of the $D_2(5'\text{-AMP})^\pm/D(5'\text{-AMP})^-$ 1:1 system decreases very much less than expected. In line herewith is the observation that with 6-dimethylaminopurine the stacking tendency is already reduced by a factor of about 1/2 by changing the solvent from water to an aqueous solution containing about 5% (v/v) dioxane (= 0.64 M dioxane) [32]. Hence, under conditions with reduced solvent polarity a coulombic contribution in the stack formation tendency of $D_2(5'\text{-AMP})^\pm/D(5'\text{-AMP})^-$ becomes evident. This result may be meaningful regarding the situation in biological systems (see section 3.5).

3.5. Concluding remarks on the self-association of adenosine monophosphates: extrapolation towards biological systems

Comparison of the acidity constants given with the equilibria given by eqs. 4–6 and 10–12 for 5'-AMP in D_2O and 50% (v/v) dioxane-D_8/D_2O, respectively, shows that the range of stability of $H(5'\text{-AMP})^-$ is extended by 0.5 pH units into the acidic range, but more importantly, by 1.2 pH units toward the alkaline range. In other words, this species is, under conditions with a reduced effective dielectric constant, expected to occur in

large amounts under physiological conditions [15]. The same trends were observed for 5′-ATP [16] for the corresponding equilibria and solvent conditions. Consequently, lowering of the effective dielectric constant, as is known to occur in the active-site cavities of enzymes [11–13], will reduce the charge of the phosphate due to the increased proton affinity and this in turn will favor stacking compared with the situation for free AMP^{2-} (see table 1 and fig. 4). Consequently self-stacking of partly protonated adenine nucleotides will have to be considered in biological systems.

There is one further aspect: we have seen that partial protonation of the adenine moieties facilitates self-stacking quite remarkably. This effect may possibly occur via an enhanced donor-acceptor (or dipole-dipole) interaction as discussed in section 3.2. Should this be the case, then there is no need to bind a proton to N-1 and introduce a whole positive charge (which, if the system is saturated, reduces stacking again); the formation of a hydrogen bond to N-1, e.g., from a suitable side chain of a protein, would be enough to favor stack formation. Indeed, hydrogen bonding is known to be favored by reduced solvent polarity [34] and 'cooperative mechanisms' for related systems have already been suggested [34,35]. In fact, such hydrogen-bond formation might even be more effective in promoting stacking, since only dipoles are initiated and the repulsion effects will thus be smaller than if whole charges are introduced. Moreover, such a stacked aggregate would also allow a fast 'transport of information': an initiated dipole at one end of the stack would also be felt at the other end.

Finally, the three examples shown in fig. 5 for the interrelations between the size of the association constant and the amounts of the stacked species formed are intended to convey an indication on the extent of stacking as a function of the concentration and the size of the association constant. It should be noted that the concentration scale of the example on the right-hand side is expanded by a factor of four compared with the other two examples. Considering that the concentration of adenine nucleotides can reach rather high values, e.g., about 0.1 M in chromaffin granules of the adrenal medulla [36–39], it is evident that self-stacking of the kind described here must be expected to occur and also to play a role in living systems.

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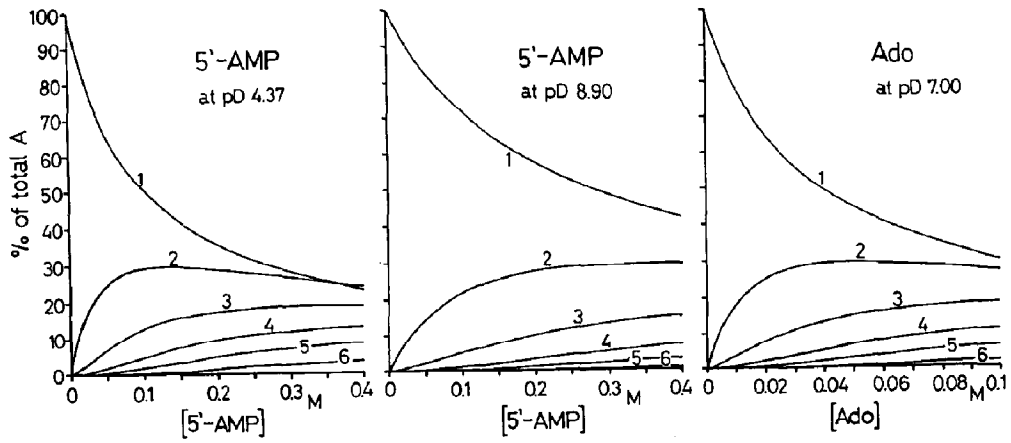

Fig. 5. Variation of the proportions of 5′-AMP or adenosine (= A) present in the monomer (1), dimer (2), trimer (3),..., and hexamer (6) in D_2O solutions at 27 °C as a function of the total concentration of 5′-AMP at pD 4.37 ($K = 5.6\ M^{-1}$ for the D(5′-AMP)$^-$/D_2(5′-AMP)$^\pm$ 1 : 1 system, see table 1; $I = 0.1$ to ~ 0.6) and at pD 8.90 ($K = 2.1\ M^{-1}$ for 5′-AMP^{2-}; $I = 0.1$ to ~ 1.2) or of adenosine at pD 7.00 ($K = 15\ M^{-1}$; $I = 0.1$).

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